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COMPARISON OF THE CHEMICAL COMPOSITION OF ALCOHOLIC EXTRACTS OF NEOPLASTIC TISSUES WITH SIMILAR EXTRACTS OF NORMAL MUSCLE[1,2]

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A preliminary report was given previously (1) on the chemical composition of alcoholic extracts of rat sarcomas following reduction in vacuo of the alcoholic content from 80 to 30 per cent. Intratumoral injection of the 30 per cent alcoholic concentrates into strictly inbred rats resulted in tumor regression in 203 of the 210 tumor-bearing rats treated. The tumor susceptibility of 149 of these cured rats was tested and it was found that all of them had developed tumor resistance. Later studies (2) showed that when the extract was injected subcutaneously into 94 rats of inbred strains, 50 per cent of the rats developed resistance to the growth of a tumor which had originated in their own inbred strain. This percentage of resistance compares favorably with that induced by vaccines in many diseases.

In view of these data a more comprehensive and systematic survey of the general composition of these extracts is reported in this paper. Moreover, a comparison will be made between the composition of such extracts from a number of neoplasms with that of identically prepared extracts of a normal mesodermal tissue from normal rats, from rats bearing the tumors, and from rats in which the tumors had been made to regress, and which were then resistant to implantation of the same tumor. Skeletal muscle, consisting of its muscle

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[2]Presented to the Biological Chemistry Division of the American Chemical Society, Chicago, September 11, 1953.

[3]Submitted in partial fulfillment of the requirements for the degree of Master of Science at the University of Pennsylvania.

fibres and connective tissue sheaths, was chosen for this purpose, since it was the host tissue in which the tumors grow.

Materials and Method

Source of extracts. Rats of two widely different, strictly inbred strains, the Aptekman albino and the Lewis albino strains, were used. Four types of 100 per cent transplantable neoplasms native in these strains were studied, namely Aptekman sarcoma No. 6, Lewis sarcomas No. 7 and No. 10, and the Lewis lymphoma No. 8. The lymphoma arose spontaneously, while the sarcomas were induced originally by means of an injection of methylcholanthrene. The tumors used for these studies were composed of compactly arranged cells, the majority of which were neoplastic (3). They had little stroma or blood, no edema fluid or hemorrhages and, with one exception, practically no necrosis. It had been shown by Lewis (4) that hydrocarbon-induced sarcomas contain muscle tissue, although in late-transplanted generations the muscle fibers seem to have become dedifferentiated or die out. When the transplantable tumors had grown to a suitable size (40 to 60 grams), they were removed from the host. After this the skeletal muscle from these rats, as well as muscle from normal and immunized rats was removed for extraction. The immunized rats had been prepared as described previously (1)(3).

Preparation of extracts. The animals were anesthesized with ether, and the tissue removed from each rat was kept in the cold room (3° to 4°C) until the total amount removed from all the rats was accumulated. Muscle was removed in the same manner from normal rats and from rats immunized (1, 3) against the growth of these sarcomas.

The weighed tissue was taken from the cold room and minced in a Waring blendor with an amount of 95 per cent ethyl alcohol equal to its weight. During the removal of the muscle, it became evident that it contained less moisture than the tumor tissue. Samples dried in the oven at 100°C indicated that tumor tissue had about 85 per cent moisture, whereas muscle had only about 72 per cent. For this reason, 13 ml. of water per 100 grams of muscle tissue were added before blending the alcoholic extracts of muscle so that the final extracts of muscle and of tumor would have the same water content.

The blended mixtures were allowed to stand in the cold room overnight and then centrifuged. Twice their volume of 95 per cent ethyl

alcohol was then added to the supernatants and after shaking the mixture was allowed to remain in the cold room for from two to ten days. The sediment, which was mainly crystalline, was centrifuged off in a refrigerated centrifuge. When tested with the alcoholometer, the alcohol content of the extracts was found to be 80 per cent. More crystals deposited in the extract on standing in the cold, and these were filtered off at 2° to 3°C. The clear extracts were then concentrated at 28° to 30°C with low pressure to one-seventh of their volume, and the final alcohol concentration was adjusted to 30 per cent. The extracts were left in the freezing box at —15°C until the maximum sedimentation occurred; then they were filtered through Whatman paper No. 42 at the same low temperature. The resulting solutions were stored at —6°C until used for analysis. They remained clear indefinitely at this temperature.

Since the major portion of the extracts was needed for chemical studies, only a small amount of each extract was utilized for treatment of one or more tumor-bearing rats, in order to assure that these tumor extracts had potency similar to those tested in earlier studies (1, 3).

Chemical Methods

All chemical analyses were carried out on duplicate or triplicate samples, and the figures given represent the averages. When colorimetric methods were used readings were made in the Klett-Summerson photoelectric colorimeter with the appropriate filters.

Solid weight determinations were made on samples dried for two hours at 110°C, and *ash* by incinerating at 450°C for one and one-half hours in platinum dishes. Inorganic and organic *phosphorus* were determined by the Fiske and Subbarow method (5); *sulfur* by the Pregl (6) gravimetric method; and *potassium* was tested for qualitatively with chloroplatinic acid.

Nitrogen was measured by the Markham method (7); *desoxyribonucleic acid* (DNA) by the Dische diphenylamine method (8), adapted to quantitative analyses by Seibert (9); and *ribonucleic acid* (RNA) by the Drury method (10), as recommended by the studies of Miller, Goldner and Miller (11).

Carbohydrate, calculated as glucose, was determined by the Koehler modification (12) of the Morris anthrone method, using a ten-minute heating period, since in all cases more color was given than by a one and a half minute heating period. *Glucose* was also studied directly

by the Somogyi modification (13) of the Shaffer-Hartman method. *Fructose* was calculated from the results obtained by the tryptophane-perchloric acid method, as recommended by Seibert, Pfaff and Seibert(14). *Sialic acid,* found in serum by Blix (15), was determined* by the Ehrlich reaction, as recommended by Werner and Odin (16).

Ascorbic acid was determined quantitatively by the Lowry, Lopez and Bessey method (17). As was shown earlier (1), it has a maximum absorption at 2600Å, which is also the maximum absorption of the tumor extract.

Lactic acid was analyzed by the Miller and Muntz (18) method.

Cholesterol, both free and combined, was measured on samples freed of alcohol, by the Shoenheimer and Sperry (19) method, and *fatty acids* by the dichromate oxidation procedure of Bloor (20) on fractions extracted with 7:1 petroleum ether-chloroform mixture (21).

Results

The analytical data on these extracts have been collected for comparative purposes in Table 1. Since the total solid content of the different extracts varied, the analytical values were recorded on a solid-weight basis in order to give a quantitative comparison between the different extracts. It shows that in certain of the constituents analyzed there is a striking contrast, even beyond the spread of individual figures, between the values for the tumor extracts and those for all the extracts of the three types of non-tumorous tissues, which are closely similar to each other.

Total solids varied from 19 to 34 milligrams per milliliter on the different extracts. The largest portion of all the extracts consisted of ash (10 to 36 per cent), amino acids or proteins (29 to 43 per cent), lactic acid (7 to 29 per cent), and RNA (6 to 17 per cent. Minor amounts of carbohydrate, DNA, ascorbic and sialic acid were also present.

Ash. Of the total solid content, 10 to 36 per cent was found to be inorganic material, and on the whole there was less in the tumor (10.4 to 20 per cent) than in the majority of the non-tumor extracts (20.2 to 35.9 per cent). About 10 per cent or less of this ash was phosphorus, and it was noteworthy that there was five to seven times as much inorganic phosphorus in the non-tumor tissue (0.91 to 2.05

*A preparation of sialic acid kindly given to us by Dr. Blix was used as standard.

TABLE 1

CONCENTRATION OF CONSTITUENTS IN EACH EXTRACT (GAMMA PER MILLIGRAM OF SOLID WEIGHT)

	Tumor					Muscle from Tumor-Bearing Rats					Muscle from Resistant Rats			Muscle from Normal Rats		
	T-I	T-II	LT-I	LT-II	LT-III	NT-I	NT-II	LNT-I	LNT-II	LNT-III	NIm-I	NIm-II	LIm-I	NN-I	NN-II	LNN
Total ash	200	185	153	104	161	218	240	311	341	317	202	271	307	300	240	359
Phosphorus*																
Inorganic*	1.2	1.8	2.6	3.1	4.0	11.2	15.5	20.5	18.5	18.0	9.1	20.6	15.9	8.6	11.1	17.1
Organic*	4.7	7.3	8.4	7.7	12.8	8.3	6.6	10.3	7.7	9.6	8.3	9.7	8.6	8.6	8.5	7.8
Carbohydrate																
Glucose (anthrone)	17	18	9	30	8	50	61	41	41	45	44	36	40	52	36	37
Glucose (copper)*	14	13	14	43	20	37	38	33	24	39	29	32	40	36	27	34
Fructose (TA)*	19	14	14	24	34	11	9	11	9	17	11	7	12	10	7	12
Sialic acid		7.8			4.2		2.2				1.9				1.9	
Ascorbic acid	0.16	0,12		0.48	3.99		0.13		0.12	0.26	0.08	0.09	0.17		0.08	0.15
DNA	5.9	6.0	4.2	5.5	11.1	2.3	1.4	2.2	1.8	3.3	1.6	1.2	1.6	1.8	1.5	1.2
RNA	78	90	108	58	124	138	143	132	122	128	166	141	119	121	153	118
Total nitrogen*	62	70	64	74	77	88	77	75	89	82	65	86	79	89	80	82
Proteins or amino acids	303	342	286	395	337	406	331	330	429	374	363	390	371	433	346	390
Lactic acid	124	119	159	71	114	274	254	255	199	254	286	277	293	283	239	266
Fatty acids	Tr.	0	Tr.	Tr.	Tr.		0					0			Tr.	
Cholesterol		0					0					0			0	
Total	728	768	719	664	763	1088	1033	1071	1134	1121	1064	1116	1132	1191	1017	1171
	Milligrams per millimeter															
Total solids	25.5	27.6	19.0	25.6	22.6	30.3	31.7	34.1	28.6	26.1	25.3	25.8	29.0	28.0	27.1	24.5

*These were not counted in the totals because they were included in other analyses.

T=tumor tissue; NT=muscle from tumor-bearing rats; NIm=muscle from immunized rats; and NN=muscle from normal rats. Prefix L indicates Lewis rats (LT-I-sarcoma No. 10; LT-II-sarcoma No. 7, and LT-III-lymphoma No. 8).

per cent) as in the tumor tissue (0.12 to 0.40 per cent). The amount of organic phosphorus (0.47 to 1.28 per cent) appeared to be in approximately the same range in all extracts. Sulfur determinations on three extracts, viz., from a tumor, from the muscle of a tumor-bearing rat, and from the muscle of an immunized rat, showed respectively 6.7, 4.5 and 6.6 per cent of the total ash as sulfur. Since the addition of barium chloride directly to the extracts caused no precipitate it would seem that the sulfur did not exist as free sulfate, but must have been in organic combination, probably in the sulfur amino acids. A positive test with chloroplatinic acid indicated the presence of potassium in the extracts.

Nitrogen constituents. The total nitrogen content varied in all extracts between six and nine per cent. It was not significantly different in the extracts of tumor tissue (6.2 to 7.7 per cent) from those of muscle tissue (6.5 to 8.9 per cent). The largest part of the nitrogen was found to be in the amino acid or protein moiety.* Some of the nitrogen was included in the nucleic acid components, and the analyses show that the desoxyribonucleic acid (DNA) was greater in all of the tumor extracts (0.42 to 1.11 per cent) than in the extracts of normal tissue (0.12 to 0.33 per cent). This is consistent with numerous reports in the literature (*22*). However, the DNA represented a low percentage of the total solids in all extracts. There was, in general, 40 to 140 times as much RNA (11.8 to 16.6 per cent) as DNA (0.12 to 0.33 per cent) in the extracts of muscle, and in contrast to this, only 5 to 14 times as much RNA (5.8 to 12.4 per cent) as DNA (0.42 to 1.11 per cent) was found in the tumor extracts. The higher content of DNA (1.1 per cent) and RNA (12.4 per cent) found in the extract of the lymphoma is consistent with the higher content of organic phosphorus (1.28 per cent) present in this extract.

Ascorbic acid was found to form only a small percentage (0.08 to 0.4 per cent) of the total solid content of the extracts.

Carbohydrate constituents. Carbohydrate in general, was determined by the anthrone method and calculated as glucose. It was shown, as indicated in Table 1, to be much less in the tumor extracts (0.8 to 1.8 per cent), except the Lewis sarcoma No. 10 (3.0 per cent), than in the muscle extracts (3.6 to 6.1 per cent). Glucose, determined by the copper reduction method was in general somewhat less than by the anthrone method, except in the extracts of the three

*This will be considered in detail in a future contribution.

Lewis tumors, where the relatively higher values may have been due to their relatively higher ascorbic acid contents, since ascorbic acid gives copper reduction.

Fructose values obtained by means of the tryptophane-perchloric acid reaction, were found to be somewhat higher in the tumor extracts (1.4 to 3.4 per cent) than in the normal tissue extracts (0.7 to 1.7 per cent). However, this contrast must be viewed with caution since the two highest tumor values (2.4 and 3.4 per cent) were obtained on the two extracts shown also to have higher ascorbic acid values, and Cohen (23) showed that ascorbic acid gives the tryptophane-perchloric acid reaction. This will be discussed later.

Sialic acid. The two tumor extracts which were analyzed for this substance by the Ehrlich reaction, showed higher values (0.78 to 0.42 per cent) than did the three muscle extracts (0.19 to 0.22 per cent).

Lactic acid. The proportion of lactic acid in the tumor extracts (7 to 16 per cent) was significantly lower than in the extracts of muscle tissue (20 to 27 per cent).

Cholesterol and fatty acids. No cholesterol, free or bound, and only traces of fatty acids or none at all, were found in either the extracts of neoplastic tissue or in those of muscle tissue.

Biological tests. The results obtained in studies of the potency of the various alcoholic extracts showed that the extracts of the sarcoma tissue caused tumor regression followed by development of tumor resistance in all of the treated animals, while the extracts of the various types of muscle failed to do so. (Table 2). Only in the case of one rat, out of 17 treated, did injections of the normal muscle extract (from a tumor-bearing rat) succeed in bringing about regression of a tumor, and when this rat was subsequently challenged it did not show resistance to the tumor. Earlier studies had shown that alcohol (30 to 50 per cent) failed to cause tumor regression (followed by tumor resistance (1) (3) (24), and, therefore, these tests were not repeated in this study.

Discussion

Among the ash constituents, phosphorus represented 3 to 11 per cent of the total ash. Of this phosphorus 20 to 28 per cent was inorganic and the remainder (52 to 70 per cent) organic phosphorus in the tumor extracts, whereas in the extracts of normal muscle 50 to

70 per cent of the total phosphorus was inorganic, and 30 to 50 per cent was organic phosphorus.

In the remaining ash sulfur and potassium were found and the sulfur appeared to exist chiefly in organic combination.

TABLE 2

POTENCY OF VARIOUS EXTRACTS IN PRODUCTION OF TUMOR REGRESSION AND TUMOR RESISTANCE

Extract used	Extract from tumor	Tumor injected into test rat	Number of injections with extract	Results of oncolytic tests	Tumor used for challenge	Result of challenge
NN-I		#6	6	Tumor grew		
		#6	8	" "		
NN-II		#6	7	Tumor grew		
		#6	9	" "		
NT-I		#6	4	Died—small tumor		
		#6	6	Tumor grew		
NT-II		#6	7	Tumor grew		
		#6	10	Tumor regressed	#6	Tumor grew
		#10 (Lewis)	5	Tumor grew		
NIm-I		#6	7	Tumor grew		
		#6	7	" "		
		#10 (Lewis)	5	" "		
NIm-II		#6	7	Tumor grew		
		#6	7	" "		
T-I	#6	#6	6	Tumor regressed	#6	No tumor grew
		#6	6	" "	#6	" " "
T-II	#6	#6	7	Tumor regressed	#6	No tumor grew
		#6	6	" "	#6	" " "
		#6	6	" "	#6	" " "
		#10 (Lewis)	5	" "	#10	" " "
None		#6	–	Tumor grew		
		#6	–	" "		
		#6	–	" "		
LN-I		#12	7	Tumor grew		
LNT-II		#12	9	Tumor grew		
LIm		#12	9	Tumor grew		
LT	#10	#12	9	Tumor regressed	#12	No tumor grew
None		#12	–	Tumor grew		
		#12	–	" "		
		#10	–	" "		
		#10	–	" "		

It is difficult to state conclusively in what form the carbohydrate exists, because the different tests available are not entirely specific. However, the results with each test were evaluated in terms of the substance giving the highest specificity and reported as such in the table.

The anthrone test, for example, will determine both glucose and fructose, and the blue green color of the solutions indicated that RNA also may have contributed something to the final values. However, this contribution must have been small, since heating for ten minutes was found to give higher results than a one and a half minute heating period, which is used for determining ribose sugars (12).

The tryptophane-perchloric acid reagent was originally recommended by Cohen (23) as a test for desoxyribonucleic acid, but results on the extracts, calculated as such, would be 6 to 10 times as much as actually found by the more specific diphenylamine reaction. Fructose and ascorbic acid also give reactions with this reagent. The results were, therefore, calculated as fructose, because in view of the values obtained for ascorbic acid with the more specific reagent of Lowry, Lopez and Bessey (17), it is obvious that only a small amount of the tryptophane-perchloric acid reacting substance in the extracts can be ascorbic acid. Since the true ascorbic acid values for the LT-II and LT-III extracts were found to be relatively high, this would account for the somewhat higher tryptophane-perchloric acid values (0.24 and 0.34 per cent) of these two fractions.

The hypothesis put forth by Blix (15) that all or much of the tryptophane-perchloric acid reacting material in serum may be sialic acid was tested on five representative extracts, as indicated in the table. Sialic acid gives practically the same degree of color reaction with the tryptophane-perchloric acid reagent as does fructose, and if the results were calculated as sialic acid, there would be 5 to 7 times too much sialic acid in the extracts, as compared with the results based on the more specific Ehrlich reaction. Therefore, it is probable that most of the substance in the extracts giving the tryptophane-perchloric acid reaction is not sialic acid, and may, therefore, be fructose, as formerly concluded.

The content of glucose was furthermore determined directly by the copper reduction method, and, with the exception of those extracts having a high ascorbic acid content, the glucose was lower in the tumor extracts than in all the muscle extracts. This, along with the

fact that lactic acid also was lower in the tumor extracts, is consistent with the view expressed in the literature (25) that tumors have a high cellular metabolism.

Although cholesterol and fatty acids have a low solubility in alcohol under the conditions used in these experiments, it is interesting that practically none was found by analyses on these extracts. It is possible that the small amount that may have been extracted, was removed by clarification of the extracts at the very low temperature used. The suggestion given, therefore, by Hauschka (26) in reference to the work of Aptekman and Lewis (2)(3)(24)(27) "that lipids and lipoproteins may indeed be basically concerned in the antigenic individuality of neoplasms is further borne out by the successful immunization of rats against transplantable sarcomas of closely corresponding genotype, following a course of injections with alcoholic tumor extract," could not be substantiated by the results obtained in the present study.

When all the constituents determined are totaled, practically 100 per cent, within experimental error, of the total solid material of the normal tissue extracts was accounted for, but it is highly significant that only about 75 per cent of the tumor extract could be identified. The differences found in these studies on alcoholic extracts of malignant and normal tissues indicate that there are gross fundamental differences in the chemical make-up of these two types of tissue and that certain well recognized components exist in different relative amounts in the two types of tissue extracts.

Summary

Alcoholic extracts were made from three rat sarcomas and one lymphoma and compared with extracts made in the same manner from the skeletal muscle of inbred normal, tumor-bearing, and tumor-resistant rats.

Analyses showed the extracts to contain 19-34 mg. per ml. of solids. Of the solids, 10-36 per cent was ash, 28-43 per cent was amino acids (based on the nitrogen content), 6-17 per cent was nucleic acids (RNA + DNA), 7-29 per cent was lactic acid, and 0.8-6 per cent was glucose. There was no cholesterol and only traces of fatty acids. Phosphorus, sulfur, and potassium were found to be present in those extracts tested.

Little variation was found in the chemical composition of normal

muscle extracts, but significant differences were noted between the tumor extracts and these normal tissue extracts in many of the components analyzed. For example, in the tumor extracts the ash was about half, the inorganic phosphorus one-fifth, lactic acid half, glucose half, RNA two-thirds, but DNA three times as much as in the normal tissue extracts.

A total of 100 per cent of the constituents of the normal tissue extracts was accounted for, whereas only about 75 per cent was identified in the case of the tumor extracts.

References

1. Lewis, M. R., King, H. D., Aptekman, P. M., & Seibert, F. B. 1948. Further studies on oncolysis and tumor immunity in rats. *J. Immunol.*, **60**, 517-28.
2. Aptekman, P. M., Lewis, M. R., & King, H. D. 1949. Tumor-Immunity induced in rats by subcutaneous injection of tumor extract. *J. Immunol.*, **63**, 435-40.
3. Aptekman, P. M., Lewis, M. R., & King, H. D. 1946. A method of producing in inbred albino rats a high percentage of immunity from tumors native to their strain. *J. Immunol.*, **52**. 77-86.
4. Lewis, W. H. 1939. Dibenzanthracene mouse sarcomas. *Am. J. Cancer*, **37**, 521-30.
5. Fiske, C. H., & Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66**, 375-400.
6. Pregl, F. 1930. Quantitative organic microanalysis. *P. Blakiston's Son & Co., Inc.*, Philadelphia.
7. Markham, R. 1942. A steam distillation apparatus suitable for micro-kjeldahl analysis. *Biochem. J.*, **36**, 790-791.
8. Dische, Z. 1930. Über einige neue charakteristische Farbreaktionen der Thymonukleinsäure und eine Mikromethode sur Bestimmung derselben in tierischen Organen mit Hilfe dieser Reaktionen. *Microchemie*, **8**, 4-32.
9. Seibert, F. B. 1940. Removal of the impurities, nucleic acid and polysaccharide from tuberculin protein. *J. Biol. Chem.*, **133**, 593-604.
10. Drury, H. F. 1948. Identification and estimation of pentoses in the presence of glucose. *Archiv. Biochem.*, **19**, 455-66.
11. Miller, G. L., Golder, R. H., & Miller, E. E. 1951. Determination of pentoses. *Analytical Chemistry*, **23**, 903-5.
12. Koehler, L. H. 1952. Differentiation of carbohydrates by anthrone reaction rate and color intensity. *Analytical Chem.*, **24**, 1576-9.
13. Somogyi, M. 1937. A reagent for the copper-iodometric determination of very small amounts of sugar. *J. Biol. Chem.*, **117**, 771-76.
14. Seibert, F. B., Pfaff, M. L., & Seibert, M. V. 1948. A serum polysaccharide in tuberculosis and carcinoma. *Archiv. Biochem.*, **18**, 279-95.
15. Blix, G. 1936. Über die kohlenhydratgruppen des submaxillarismucins. *Z. physiol. Chem.*, **240**, 43-54.
16. Werner, I., & Odin, L. 1952. On the presence of sialic acid in certain glycoproteins and in gangliosides. *Acta. Soc. Med. Upsaliensis. Upsala Läkareforenings Förhandlingar*, **57**, 230-41.
17. Lowry, O. H., Lopez, J., & Bessey, O. A. 1945. The determination of ascorbic acid in small amounts of blood serum. *J. Biol. Chem.*, **160**, 609-15.

18. MILLER, B. F., & MUNTZ, J. A. 1938. A method for the estimation of ultramicro quantities of lactic acid. *J. Biol. Chem.*, **126**, 413-21.

19. SHOENHEIMER, R., & SPERRY, W. M. 1934. A micromethod for the determination of free and combined cholesterol. *J. Biol. Chem.*, **106**, 745-760.

20. BLOOR, W. R. 1947. A colorimetric procedure for small amounts of fatty acid. *J. Biol. Chem.*, **170**, 671-674.

21. HAVEN, F. L., BLOOR, W. R., & RANDALL, C. 1949. Lipids of the carcass, blood plasma and adrenals of the rat in cancer. *Cancer Res.*, **9**, 511-14.

22. ZAMECNIK, P. C. 1952. The biochemistry of neoplastic tissue. *Ann. Rev. Biochem.*, **21**, 411-30.

23. COHEN, S. S. 1944. Observations on the estimation of desoxyribose nucleic acid. *J. Biol. Chem.*, **156**, 691-702.

24. APTEKMAN, P. M., & LEWIS, M. R. 1951. Evidence of resistance to tumor graft in early stages of induced oncolysis. *J. Immunol.*, **66**, 361-4.

25. WARBURG, O. 1930. The metabolism of tumors. *Dickens, F. Trans. Constable and Co. Ltd.* London, England.

26. HAUSCHKA, T. S. 1952. Immunologic aspects of cancer. *Cancer Res.*, **12**, 615-33.

27. LEWIS, M. R., APTEKMAN, P. M., & KING, H. D. 1949. Inactivation of malignant tissue in tumor-immune rats. *J. Immunol.*, **61**, 321-6.

Growth, 1954, **18**, 13-26.

MATERNAL HYPO- AND HYPERTHYROIDISM IN ALBINO RATS AS INFLUENCING GROWTH, METABOLISM AND ENDOCRINE GLAND WEIGHTS OF THE OFFSPRING[1]

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Investigations dealing with prenatal influences of the maternal thyroid gland activity upon that of the offspring have been extremely few and those which have appeared are contradictory. In view of this paucity of evidence it is necessary to compare data across species and group lines, recognizing, of course, that wide species differences exist in the response to the various hormones.

With respect to maternal hypothyroidism during gestation, one group of data indicates that the offspring are hypothyroid while a number of other investigators report that the offspring are hyperthyroid.

Rickey (1925), in one of the earliest reports, has noted that the offspring of thyroidectomized female rats were from ½ to 1/3 smaller at birth than the control rats and remained smaller and weaker than the control rats throughout their lives. She supported her contention of hypothyroidism in the offspring by noting that feeding them desiccated thyroid substance altered their development and behavior toward that of the normal rats.

Marza and Marza (1929), using guinea pigs, report that the offspring of thyroidectomized females were smaller and weaker than the control animals. Histologically, the cells of the thyroid glands were "greatly retarded." Marza-Rusnac (1929) confirmed these findings. Contrary to this, Ukita (1919) reports that rabbits, thyroidectomized during the seventh to tenth day of pregnancy, gave birth to offspring

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whose thyroid glands were heavier than those of the control animals. Histological examination of these glands showed them to be abnormally well developed with regular large follicles containing large amounts of colloid.

McCartney and Schaffner (1949a) report that hypothyroid dams produce chicks which are hypothyroid. Other work substantiates this (McCartney and Schaffner 1949b, Wheeler and Hoffman 1948a, b and 1949). Hypothyroidism was produced in the dams by feeding thiouracil. The result that hypothyroid dams produced hypothyroid chicks might well be explained by the direct action of thiouracil included within the egg at the time of shell formation upon the embryonic thyroid. It seems unlikely that the lack of maternal thyroid activity, *per se*, could effect a change in the embryo which is only at about the gastrula stage at the time of egg laying.

Gumbreck (1950) has pointed out that the relationship between the mother and fetus is somewhat comparable to that in parabiosis between thyroidectomized male and normal female rats. He reports that the thyroid glands of many, but not all, of the normal females were enlarged. The enlargement indicates excessive stimulation by thyrotropic hormone from the pituitary of the male. If the relationship of parabiotic animals is to be considered as being analogous to the maternal-fetal relationship, the placenta would necessarily be permeable to thyrotropic hormone. Tobin (1941) has found that the thyroid glands of rat fetuses show no signs of stimulation by injections of thyrotropic hormone. It is surprising that the hemoendothelial placenta of the rat, consisting of only a fetal endothelium between the maternal and fetal blood supplies would be an effective barrier against the passage of a molecule even as large as that of the thyrotropic hormone (Windle 1940). It is possible that the fetal thyroid of the rat does not respond to the thyrotropic hormone.

The effects of maternal hyperthyroidism upon the metabolic activity of the offspring are no less confusing. Reports have been published which indicate that the offspring of hyperthyroid female rats are, themselves, hyperthyroid while others, on equally good evidence, conclude that maternal hyperthyroid rats produce hypothyroid offspring.

Rickey (1925) reports that the behavoir of offspring of hyperthyroid female rats is very similar to that of animals made hyperthyroid by feeding desiccated thyroid substance. Dispensa and Hornbeck (1941) also claim to have produced rats which were hyperthyroid, as

judged by their growth records, by feeding desiccated thyroid substance to mothers during pregnancy.

On the other hand, Cunningham (1941) and Morrison and Cunningham (1941) claim that feeding desiccated thyroid to pregnant female rats produces a hypothyroid condition in the offspring, this being manifest by reduced growth rate, and that feeding desiccated thyroid to the offspring increased the growth rate to normal. Rose's (1947) findings, that fetuses removed from hyperthyroid female rats tend to have their maturation delayed, substantiate these claims. On the basis of these investigations there would seem to be considerable variation in the balance of hormonal control of fetal thyroid activity between different species, different strains, or perhaps different individuals.

The present study is intended to provide additional information about the relation of maternal hypo- and hyperthyroidism to the growth and metabolism of the offspring.

Methods and Conditions

Subjects

The subjects of the P (parent) generation were 80 female and 10 male albino rats between 90 and 120 days of age at the start of the experiment.

The females of the P generation were divided into three groups. Thirty-one of the animals were designated as the $Hypo_P$ group. The $Hyper_P$ group had 22 animals while the remaining 27 animals served as the $Control_P$ group.

The $Hypo_P$ Group. The 31 female rats of the $Hypo_P$ group were rendered hypothyroid by surgical removal of part or all of the thyroid gland. The operative technique was essentially as described by Ingle and Griffith (1949). Seven of the animals of this group were totally thyroidectomized and in each instance an attempt was made to implant at least two parathyroid glands in the sternohyoid muscle. No check was made on the success of these implants. Close observation of these thyroidectomized animals failed to reveal signs of parathyroid deficiency. Twelve animals had one lobe of the thyroid gland completely removed and about ½ of the other lobe, care being taken to leave one parathyroid gland in place and undisturbed. The remaining twelve rats had only one lobe of the thyroid gland removed. Four weeks after thyroidectomy the basal metabolic rate of the $Hypo_P$

group was determined. (This procedure is described in detail later). At the completion of this test they were placed in breeding cages with the males.

The Hyper$_P$ Group. The 22 females of this group were bred at the same time and to the same males as the Hypo$_P$ females. Vaginal smears were taken each morning and examined for the presence of sperm. Where sperm was found the animal was isolated and six days later injections of thyroxine started. Crystalline thyroxine, obtained from the Squibb Pharmaceutical Company, was prepared for injection as described by Monroe and Turner (1946). The thyroxine was dissolved in a 0.01N solution of sodium hydroxide and then diluted to 0.005N. Since the body weights of the animals were fairly uniform, ranging from 190 to 220 grams, it was decided to administer constant amounts of thyroxine. Six females received daily injections of 0.012 mg. of thyroxine, six, 0.025 mg., five, 0.050 mg., and five, 0.075 mg. The smallest dose is about 2.1 times the amount normally secreted by the thyroid glands of pregnant female rats of this weight while the largest is about 13.4 times that amount (Monroe and Turner 1946). The injections were stopped two days prior to the expected date of delivery to insure normal delivery of the young (Rose 1947). Two weeks after weaning the young the females of the P generation again received daily injections of thyroxine. One week after the start of the injections the basal metabolic rate of the group was determined.

The Control$_P$ Group. The basal metabolic rate of this group was determined at the same time as that of the Hypo$_P$ group. They were placed in the same breeding cages and allowed to mate at the same time as the members of the experimental groups. No sham operations or placebo injections were given to the animals of this group.

The F_1 Generation. Immediately after birth of the F_1 generation (offspring of the P generation) the litter size was reduced to a maximum of six animals. Those discarded were selected at random from the litter. This was done to avoid possible inanition in the case of the larger litters. The F_1 generation was weaned at four weeks of age.

Growth.—During the gestation and nursing period the mothers (P generation) were fed the standard laboratory diet of Purina Laboratory Chow supplemented weekly with greens and a small amount of raw hamburger. After weaning, the animals of the F_1 generation were fed Purina Laboratory Chow *ad libitum.* Each litter was weighed at one week of age and weekly thereafter for the next 11 weeks. The mean

weights of the litters of the three groups, regardless of sex, were recorded. At 13, 24, 26 and 33 weeks of age the members of the three groups were weighed individually.

Food Consumption.—Wang (1923) has suggested that an index of the total metabolism of an animal may be obtained by measuring its daily food consumption. The measure of daily food consumption was started at 13 weeks of age. Water was available at all times. Throughout the food consumption measures the standard diet of Purina Laboratory Chow, in powdered form, was presented in food cups especially designed to prevent scattering of the food. Occasional examination of the cages revealed no evidence of spilled food particles. The food cups were weighed and filled daily. A mean of the amounts of food consumed on three consecutive days was taken as an index of the daily food consumption.

Basal Metabolism.—Two respirometers of the type described by Morrison (1947) were used to measure the oxygen consumption rates. The animal chambers were immersed in a constant temperature water bath maintained at 28° ± 0.50 centigrade. Benedict and MacLeod (1929) have found this to be the critical temperature above and below which metabolic rate rises.

Following the procedure for the measurement of the basal metabolic rate of rats described by Horst, Mendel and Benedict (1934), all measurements were made between the hours of 10:00 A.M. and 4:00 P.M. Thirty minute tests were given each animal on four alternate days. A habituation period of 30 minutes was allowed prior to the actual test period. During this time the system reached equilibrium and the animal became quiet.

The basal metabolic rates were computed in terms of Calories per kilogram of body weight per hour. In computing the basal metabolism a calorific value of oxygen of 4.825 Calories per litter was assumed (Hawk *et al.*, 1947).

The animals of the F_1 generation were 26 weeks old when the metabolism tests were made. All animals had been deprived of food 24 hours prior to the test period.

Glandular Weights.—At 33 weeks of age most of the animals of each group were sacrificed, weighed and the pituitary, thyroid and adrenal glands, the gonads and seminal vesicles removed. The connective tissue surrounding the glands was carefully removed prior to weighing.

RESULTS

The P Generation

The Basal Metabolism. The influence of the thyroid ablation and injections upon the basal metabolic rates of the P generation is presented in Table 1. It is of interest to note that the mean rate of the $Hypo_P$ group was 20.4 per cent below that of the $Control_P$ group while the mean of the $Hyper_P$ group was 16.1 per cent above the mean of the $Control_P$ group. Both of these values are well beyond the normal variation of ± 7 per cent reported by Meyer and Ranson (1945). Testing the difference between the mean basal metabolic rates by the t-ratio showed both experimental groups to be different from the control group by far greater than the 1 per cent level of confidence.[2]

TABLE 1

THE BASAL METABOLIC RATES OF THE P GENERATION FEMALES IN CALORIES PER KILOGRAM OF BODY WEIGHT PER HOUR

	$Hyper_P$ Group N = 22	$Control_P$ Group N = 27	$Hypo_P$ Group N = 31
Mean	7.26	6.25	4.97
σ	0.664	0.434	0.577
σ_m	0.145	0.085	0.105
$\frac{\text{Diff.}}{\sigma d_m}$	6.01*		9.48*

*Significant at the 1 per cent level of confidence.

The mean rate of 6.25 Calories per kilogram of body weight per hour for the $Control_P$ group is very close to the 6.13 Calories per kilogram of body weight per hour reported by Carr and Krantz (1949) using the Haldane open circuit method of obtaining the oxygen consumption rate.

The F_1 Generation

Growth. Figure 1 presents the weights of the F_1 generation from one week after birth through the twenty-sixth week of age. During the first seven weeks of life the weights of these animals were extremely close. At the eighth week the $Hypo_{F1}$ group showed a consist-

[2]A method of expressing the probability of differences between groups resulting from chance variation of the measures. Thus the 1 per cent level of confidence indicates that such a difference might result from change fluctuations in the scores only once in every 100 such samples. The other 99 times would be as a result of the experimental variable.

ently lower mean weight which persisted throughout the measurements. Not until the thirteenth week did the animals of the $Hyper_{F1}$ group show a slight increase weight over the $Control_{F1}$ group. Neither of these differences was statistically significant.[3]

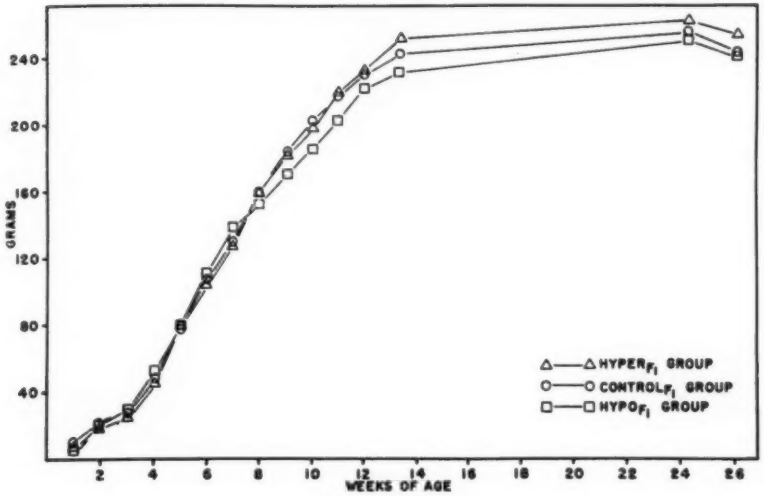

FIGURE 1

Growth curves, as represented by body weight, of the F_1 generation.

At 13, 24, 26 and 33 weeks of age individual weights were obtained, making possible an analysis of the weights with respect to the sex of the animals. Application of analysis of variance to these data yielded a significant F ratio between the females of the three groups at 13 weeks of age. Testing the difference between the means of the t-ratio showed that the females of the $Hyper_{F1}$ group weighed very significantly (1 per cent level of confidence) more than the $Control_{F1}$ females. No other differences between the three groups of either sex gave significant F ratios.

Rate of Food Consumption. Wang (1923) has suggested that an index of the total metabolism of an animal may be obtained by measuring its daily food consumption. The F ratios of the analysis of

[3]When a difference between groups may be expected to occur less frequently than five times in every 100, the difference is considered statistically significant.

variance used to compare the mean grams of food consumed per kilogram of body weight per 24 hours of the three groups of either sex were not significant.

TABLE 2

MEAN BODY WEIGHT OF THE F_1 GENERATION IN GRAMS AT 13, 24, 26 AND 33 WEEKS OF AGE

Group	N	13 Weeks Mean Weight	N	24 Weeks Mean Weight	N	26 Weeks Mean Weight	N	33 Weeks Mean Weight
				Males				
$Hyper_{F1}$	17	292.9	17	301.1	17	285.9	10	334.0
		*28.0**		*49.2*		*52.6*		*26.6*
$Control_{F1}$	19	285.7	16	298.2	15	281.3	11	319.3
		44.1		*42.1*		*43.1*		*75.0*
$Hypo_{F1}$	11	273.5	11	304.7	10	296.3	10	319.3
		25.1		*26.4*		*28.7*		*53.1*
				Females				
$Hyper_{F1}$	14	209.5	13	224.2	13	214.3	9	230.9
		9.7		*41.2*		*41.2*		*20.1*
$Control_{F1}$	19	193.3	19	217.1	18	210.4	10	229.3
		18.1		*54.0*		*33.3*		*35.7*
$Hypo_{F1}$	12	183.2	11	202.9	11	187.6	11	214.7
		16.6		*15.0*		*11.5*		*13.8*

*Standard Deviation.

TABLE 3

MEAN GRAMS OF FOOD CONSUMPTION PER KILOGRAM OF BODY WEIGHT PER 24 HOURS OF THE F_1 GENERATION. AGE 13 WEEKS

Group	Males N	Males Mean	Males σ*	Females N	Females Mean	Females σ*
$Hyper_{F1}$	17	70.10	8.95	14	84.20	13.75
$Control_{F1}$	19	72.48	11.62	19	82.06	20.06
$Hypo_{F1}$	11	82.70	21.01	12	78.70	7.25

*Standard Deviation.

Comparison of the males and females of each group yielded a significant F. The t-test showed that only the females of the $Hyper_{F1}$ group consumed significantly more food than the males of that group.

Basal Metabolic Rate. Table 4 presents a summary of the mean basal metabolic rates of the three groups in terms of Calories per kilogram of body weight per hour. Comparison of the groups by analysis of variance showed that no significant differences existed

between the groups of either the males or females. When the males and females of each group were compared, the F ratio obtained was highly significant. The t-test showed that the females of each of the three groups had a very significantly (1 per cent level of confidence) higher basal metabolic rate than the corresponding males.

TABLE 4

BASAL METABOLIC RATE OF THE F_1 GENERATION IN CALORIES PER KILOGRAM OF BODY WEIGHT PER HOUR. AGE 26 WEEKS

Group	Males			Females		
	N	Mean	σ*	N	Mean	σ*
$Hyper_{F1}$	17	5.42	0.72	13	6.66	0.79
$Control_{F1}$	15	5.45	0.73	18	6.09	0.72
$Hypo_{F1}$	10	5.16	0.48	11	6.43	0.94

*Standard Deviation.

Gland Weight. The mean gland weights in milligrams per kilogram of body weight are shown in Table 5. Analysis of variance showed that no statistically significant differences existed between the gland weights of the three groups of males. In the case of the females it was found that the adrenal glands and ovaries of the three groups were significantly different. Examination of these differences by the t-test showed that the adrenal glands of the $Hyper_{F1}$ females and the $Hypo_{F1}$ females were reliably heavier (at the 5 and 1 per cent levels of confidence, respectively) than those of the control females. The ovaries of the $Hypo_{F1}$ group were reliably heavier than those of the $Control_{F1}$ group at the 1 per cent level of confidence.

DISCUSSION

Comparison of the basal metabolic rates of the P generation demonstrated conclusively that these animals were from different populations with respect to metabolism. It should be remembered that these values were obtained on non-pregnant females. Danforth and Loumos (1936) have shown that, using a criterion of oxygen consumption, pregnant rats are more tolerant to desiccated thyroid than non-pregnant rats. While the effects of pregnancy probably reduced the rate of oxygen consumption in the $Hyper_P$ group from that shown in Table 1, the fact still remains that the young gestated in an environment in which there was from 2.1 to 13.4 times the amount of

thyroid hormone provided by the thyroid gland of normally pregnant rats.

The growth rates of the three groups, as indicated by weight increments, prior to 13 weeks of age failed to reveal any significant

TABLE 5

MEAN GLAND WEIGHT IN MILLIGRAMS PER KILOGRAM OF BODY WEIGHT. AGE 33 WEEKS

	N	Pituitary	Thyroid	Adrenal	Gonads	Seminal Vesicles
			Males			
$Hyper_{F1}$	10	33.74	51.08	103.72	8410.4	2251.6
		*3.50**	*5.34*	*29.90*	*1194.0*	*794.5*
$Control_{F1}$	11	32.75	59.56	127.19	10140.5	2413.5
		6.75	*14.33*	*39.05*	*2107.0*	*551.5*
$Hypo_{F1}$	10	38.08	64.55	142.97	9594.1	2715.3
		6.04	*14.40*	*41.50*	*2099.0*	*779.8*
			Females			
$Hyper_{F1}$	9	66.31	70.16	233.41	239.73	
		11.22	*11.97*	*31.81*	*36.10*	
$Control_{F1}$	10	60.09	62.06	193.46	230.96	
		8.78	*33.18*	*32.77*	*48.20*	
$Hypo_{F1}$	11	63.43	78.22	236.28	307.13	
		13.43	*18.25*	*22.45*	*54.05*	

*Standard Deviation.

differences. This is at variance with the results of other studies. It will be recalled that Rickey (1925) reported a continued retardation in the growth of offspring of hypothyroid females while she and Dispensa and Hornbeck (1941) report accelerated growth in the offspring of females fed desiccated thyroid substance. Cunningham (1941) and Morrison and Cunningham (1941), on the other hand, report that feeding desiccated thyroid to pregnant females retards the growth of the offspring. The differences between the data of this study and those reported by others does not appear to be readily explained except by the kind and quantity of thyroid substance administered and possibly, by strain differences in the rats used.

When the sexes were considered separately at 13 weeks of age, the females of the $Hyper_{F1}$ group were found to be very significantly heavier than those of the $Control_{F1}$ group. At 24, 26 and 33 weeks of

age no significant differences were found. One possible interpretation of the difference at 13 weeks and the lack of significance found at the other periods is that the effects of altered metabolic environment during gestation may have produced a change in the metabolic activity of the organisms which is, in turn, reflected in the growth rate. The change in metabolic rate during this period, if it occurs, must be of a transitory nature for the subsequent course of growth changes, relative to the $Control_{F1}$ group, and the basal metabolic rates taken at 26 weeks of age showed no difference between the groups. This explanation is consistent with Peruzzi's (1937) recognition that there are certain periods in the development of an organism during which added demand is placed upon the endocrine system, particularly the thyroid gland. Acceptance of this explanation would necessitate the added assumption of a differential effect of a hyperthyroid environment during gestation upon the sexes as opposed to the effects of a hypothyroid environment during gestation.

The fact that no statistically significant differences between the groups were found in the measures of daily food consumption at 13 weeks of age is opposed to an explanation of the body weight differences based upon a transitory change in metabolism.

There is some discrepancy between the rate of food consumption as a measure of metabolism and the basal metabolic rate as measured by the rate of oxygen consumption. In comparing the males and females within each group only the females of the $Hyper_{F1}$ group consumed significantly more food than the males. No differences were noted between the males and females of either the $Control_{F1}$ or $Hypo_{F1}$ groups. Since Meyer and Ranson (1945) and Monroe and Turner (1946) as well as the present study indicate that females have a higher basal metabolism than the males, one would expect to find that the females of the $Control_{F1}$ and $Hypo_{F1}$ groups would also consume more food per kilogram of body weight than the males. This expectation was not realized.

No statistically significant differences were noted in the basal metabolic rate of the three groups at 26 weeks of age. Examination of the sex differences obtained showed the males to have a significantly lower rate than the females. These differences are consistent with the report of Monroe and Turner (1946) that females have a higher thyroid secretion rate than males when computed independently of body weight. Benedict and MacLeod (1929) failed to find

a difference in the oxygen consumption of males and females. This might result from differences in the method of computing metabolic activity used in the studies. Benedict and MacLeod computed metabolic rates in terms of square meters of body surface, while in this study the basal metabolic rate was computed in terms of kilograms of body weight. The latter technic has been shown to be the more reliable of the two (Horst, *et al.* 1934).

Comparison of the weights of the various glands of internal secretion taken at 33 weeks of age showed the adrenal glands of both the $Hypo_{F1}$ and $Hyper_{F1}$ females and the ovaries of the $Hypo_{F1}$ group were significantly heavier than those of the $Control_{F1}$ group. The lack of statistically significant differences in the weights of the thyroid glands of the three groups is in agreement with the tests of oxygen consumption. Several studies, discussed earlier, have indicated changes in thyroid gland activity of offspring of hypo- and hyperthyroid guinea pigs and rabbits. These data, however, were obtained within a few days of birth. It is conceivable that the thyroid glands of the rats of this study were either hyper- or hypoactive during this period. If such was the case it undoubtedly was of a transitory nature and certainly was not reflected in the mean body weights taken at one week of age.

In general, this study indicates that no change results in the offspring, on the basis of the measures used, when the maternal rats are either hyper- or hypothyroid during pregnancy. There may have been effects of the experimental conditions prior to one week of age but certainly none which would affect body weight after that time.

Summary

The growth, metabolism and endocrine gland weights of offspring (F_1 groups) of hypothyroid (basal metabolic rate, —20.4 per cent) and hyperthyroid (basal metabolic rate, +16.1 per cent) albino rats were compared with those of normal rats. The major findings and conclusions are as follows:

1. No differences were noted in the growth, as indicated by body weight increments, from 1 week to 33 weeks of age except at 13 weeks of age when the $Hyper_{F1}$ females weighed more than the $Control_{F1}$ females.

2. General metabolism, as indicated by the rate of food consump-

tion per kilogram of body weight, did not differ significantly in the three groups at 13 weeks of age.

3. Basal metabolism, as indicated by the rate of oxygen consumption per kilogram of body weight per hour, did not differ significantly in the three groups at 26 weeks of age.

4. Comparison of the pituitary, thyroid and adrenal glands, the testes, ovaries and seminal vesicles at 33 weeks of age showed that only the adrenal glands of the $Hypo_{F1}$ and $Hyper_{F1}$ females and the ovaries of the $Hypo_{F1}$ were significantly heavier than those of the control groups. No explanation is offered for these differences.

References

1. Benedict, F. G., & MacLeod, G. 1929. Heat production in the albino rat. II. Influence of environmental temperature, age, and sex; comparison with basal metabolism of man. *J. Nutrition,* **1**, 367-398.
2. Carr, C. J., & Krantz, J. C. 1949. Metabolism. In Farris, E., & Griffith, J. Q. The Rat in Laboratory Investigation. (2nd Ed.) Philadelphia: J. B. Lippincott Co.
3. Cunningham, B. 1941. Experimental production of cretinoid progeny by hyperthyroidization of pregnant rats. *J. Elisha Mitchell Sci. Soc.*
4. Danforth, D. N., & Loumos, S. 1936. Effect of administration of desiccated thyroid during pregnancy in the albino rat. *Proc. Soc. Exp. Biol. & Med.* **34**, 870-872,
5. Dispensa, J., & Hornbeck, R. T. 1941. Can intelligence be improved by prenatal endocrine therapy? *J. Psychol.,* **12**, 209-224.
6. Gumbreck, L. G. 1950. Personal Communication.
7. Hawk, P. B., Oser, B. L., & Summerson, W. H. 1947. Practical Physiological Chemistry. (12th Ed.). Philadelphia: The Blakiston Co.
8. Horst, K., Mendel, L. B., & Benedict, F. G. 1934. The influence of previous exercise upon metabolism, the rectal temperature and the body composition of the rat. *J. Nutrition,* **7**, 251-275.
9. Ingle, D. J., & Griffith, J. Q. 1949. Surgery of the Rat. In Farris, E. J., & Griffith, J. Q. The Rat in Laboratory Investigation. (2nd Ed.). Philadelphia: J. B. Lippincott Co.
10. McCartney, M. G., & Schaffner, C. S. 1949a. The metabolic rate and thyroid size of chicks from dams with altered metabolism. *Endocrinology,* **45**, 396-402.
11. McCartney, M. G., & Schaffner, C. S. 1949b. Chick thyroid size and incubation period as influenced by thyroxine, thiouracil and thyroprotein. *Poultry Sci.,* **28**, 223-228.
12. Marza, E., & Marza, V. 1929. Influence de la thyroidectomie des parents sur le poids de la thyroide des descendants. *Compt. rend. soc. Biol., Paris,* **101**, 234-236.
13. Marza-Rusnac, E. 1929. Recherches experimentales sur l'héredité thyroidienne. *Rev. franç d'Endocrine.,* **7**, 320.
14. Meyer, A. E., & Ranson, V. G. 1945. The metabolism of rats after thyroidectomy or during thiouracil treatment, and the effect of thyroid feeding. *Endocrinology,* **36**, 256-265.
15. Monroe, R. A., & Turner, C. W. 1946. Thyroid secretion rate of albino rats during growth, pregnancy and lactation. *Mo. Agric. exp. sta. res. Bull.,* 403.

16. MORRISON, P. R. 1947. An automatic apparatus for determination of oxygen consumption. *J. Biol. Chem.*, **169**, 667-679.

17. MORRISON, G. W., & CUNNINGHAM, B. 1941. Characteristics of the conditioned response in cretinous rats. *J. comp. Psychol.*, **31**, 413-426.

18. PERUZZI, P. 1937. Hormonal relation to respiration in vitro of some tissues during the postnatal development of the albino rat. *Arch. fisiol.*, **37**, 334-357. (Chemical Abstracts 32:9487).

19. RICKEY, E. 1925. The thyroid influence on the behavior of the white rat. *Comp. Psychol. Monogr.*, **2**, 1-76.

20. ROSE, D. 1947. Comparisons of fetal development in normal and hyperthyroid rats. *J. comp. physiol. Psychol.*, **40**, 87-105.

21. TOBIN, C. E. 1941. The effects of thyrotropic hormone on the rat. *Proc. soc. exp. Biol. & Med.*, **48**, 592-595.

22. UKITA, T. 1919. On the influence of complete thyroidectomy during pregnancy upon the development of the fetus and on the duration of gestation. *Acta Scholae Med. Univ. Imp. in Kioto*, **3**, 287-297.

23. WANG, C. H. 1923. The relation between "spontaneous" activity and estrous cycle in the white rat. *Comp. Psychol. Monogr.*, **2**, 1-27.

24. WHEELER, R. S., & HOFFMANN, E. 1948a. Goiterous chicks from thyroprotein-fed hens. *Endocrinology*, **42**, 326-328.

25. WHEELER, R. S., & HOFFMANN, E. 1948b. Influence of quantitative thyroprotein treatment of hens on length of incubation period and thyroid size of chicks. *Endocrinology*, **43**, 430-439.

26. WHEELER, R. S., & HOFFMANN, E. 1949. Goiterogenic action of iodide and the etiology of goiters in chicks from thyroprotein-fed hens. *Proc. soc. exp. Biol. & Med.*, **72**, 250-254.

27. WINDLE, W. G. 1940. Physiology of the Fetus. Philadelphia: W. B. Saunders.

Growth, 1954, **18**, 27-35.

THE EFFECT OF HIGH ALTITUDE ON THE GROWTH OF TURKEYS

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The White Mountain High Altitude Research Station, Big Pine

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The diminished partial pressure of oxygen encountered at high altitude results in the alteration of many physiological processes (11, 14). There are indications in the literature that the growth of animals may be depressed when exposed to these conditions. Hurtado (6) has reported a reduction in body weight of young Andean natives as compared to sea level residents of similar racial stock. Van Liere (13) observed that the majority of a group of young adult guinea pigs exposed to simulated altitudes of 14-18,000 feet suffered considerable weight loss. Moore and Price (10) found that rats born at 14,000 feet had a depressed growth rate, although those born at 9,600 feet or those brought to the higher altitude after weaning grew normally.

The recent availability of a high altitude station in the White Mountains of California (4), with laboratories at 10,500 and 12,500 feet, has given us the opportunity of studying the effects of high altitude on the growth and development of birds. Broad Breasted Bronze Turkeys (Meleagris gallopavo) were selected for these studies since stocks with known growth characteristics were available. Also considered were the turkey's fast rate of growth and complex nutritional requirements which might be upset by the high altitude.

Method

Thirty poults were taken from Davis (altitude 50 feet) on the day of hatching (20 April 1953) to the White Mountain Station in a conventional poult shipping box. The laboratory at 10,500 feet elevation was selected as a brooding site to minimize the anticipated interference with growth.

A brooder was made from a kerosene incubator with a run (a 3′ x

4′ x 8′ wire cage) connected to the open door. Brooding started 36 hours after the poults' removal from the incubator, and was terminated three weeks later. The poults were then kept in the wire cage in the laboratory (60°F) until 55 days old, at which time they were moved to an outside pen. An enclosed roost furnished night shelter for the birds. In the fall, when the birds were 6 months old, lights were installed in the shelter to furnsh 14 hours of illumination per day.

The turkeys were fed practical rations, mash and pellets, which had been prepared for use by the departmental flock at Davis. The composition of these rations corresponds to Ration 3, table 10, and Ration 1, table 11, as described by Asmundson and Kratzer (1). Body weights were obtained at intervals, and some birds were sacrificed to determine the weights of various organs.

A comparison of the weather at the White Mountain station and at the Davis campus for the period reported here (April-October 1953) is given in table 1.

Results

The behavior of the White Mountain turkeys indicated that they suffered some physiological disability at 10,500 feet altitude. They tended to remain in a close group, moving only slowly and intermittantly around the pen; they showed no inclination to exercise their wings. Similar birds at Davis appeared to be more active, being continuously in motion and flying over fences at an early age. When the White Mountain birds were handled (for weighing, etc.) their brief struggling induced a marked hyperventilation which persisted for a minute or two.

The mortality up to 6 months, excluding two killed accidentally, one killed for perosis, and nine sacrificed for organ weights, was 23%. All deaths occurred on the third and fourth days of brooding. Since the husbandry conditions were not optimum, these deaths may be considered a brooding failure as well as due to the high altitude. A reasonable mortality under optimum brooding conditions would approximate 10%. Moore and Price (10) found a much higher mortality (82%) in rats born at 14,260 feet, but observed no mortality in rats born at 9,600 feet or brought to the higher elevation after weaning.

The growth of the White Mountain birds was less than that of sea

TABLE 1

1953 Weather Data by Months: Davis (50 Feet Elevation) and White Mountain (Lower Station, 10,500 Feet Elevation)

	Average Temperature Max. °F.		Average Temperature Min. °F.		Extreme Temperature Max. °F.		Extreme Temperature Min. °F.		Mean 0800 R. H. (%)		Precipitation (Water & Snow) as inches water	
	Davis	W. M.	Davis	W. M.	Davis	W. M.	Davis	W. M.	Davis	W. M.	Davis	W. M.
April	71.9	37.7	43.1	21.7	90	53	29	—2	72.9	45.2	2.25	1.65
May	73.3	38.0	46.7	20.2	85	54	38	7	67.2	58.2	.35	1.78
June	83.7	54.7	52.6	35.3	99	65	44	21	64.7	34.2	.47	.30
July	98.0	65.9	55.1	49.3	105	72	49	43	68.0	43.1	.00	2.51
Aug.	89.3	64.0	54.8	43.7	102	70	49	31	77.3	34.4	.08	.00
Sept.	91.3	58.9	55.7	41.0	106	65	45	30	77.4	37.4	trace	.46
Oct.	79.1	46.7	47.1	29.9	94	55	38	9	62.7	46.2	.16	.66

Mean barometric pressure at White Mountain: 515.6 mm. Hg.

level controls during their first few weeks at high altitude. Between the time these birds were removed from the incubator at Davis and put in the brooder at White Mountain, they lost an average of 15% of their body weight. After two weeks brooding, their average weight was only 64% of that of a control group of the same hatch brooded at Davis. Beyond 11 weeks, however, the growth of the White Mountain turkeys was comparable to that expected of birds raised at sea level. A growth curve of these birds compared with standard growth data (1) and with another group raised simultaneously at Davis is presented in figure 1. The body weights represent at least 5 ♂ and 8 ♀ through the 114 day period. The 186 day weights are on 3 birds only, 2 ♂ and 1 ♀; eight of the 11 birds retained after 114 days age were killed by a predator at 6 months. The two surviving males had been the heaviest ones, and the female about average, at earlier weight periods.

The normal growth of the White Mountain birds, in spite of an early growth depression, may result from the influence of several factors which compensated any growth inhibiting effect of the high altitude. The cooler weather at White Mountain (table 1) is generally favorable to the growth of turkeys. The minimal physical activity observed in the White Mountain birds would favor a more efficient conversion of feed to body substance.

The sanitary conditions of the White Mountain turkey yard also may have enhanced the growth of birds raised there. It has been found (3) that birds raised in new areas show no growth response to antibiotic feeding, and they grow at about the same rate as antibiotic fed animals in previously used areas. This indicates that growth enhancement by antibiotic feeding results from the suppression of organisms, detrimental to the bird, which tend to "accumulate" in areas of poultry husbandry and which are absent in new areas. Excreta samples and preserved gut sections from sacrificed birds were found to be free from intestinal parasites by Prof. J. R. Douglas, School of Veterinary Medicine, Davis. This is perhaps to be expected since the White Mountain birds had not been exposed to other turkeys since hatching.

Maturation, as well as growth, appeared to be normal in the high altitude birds. The one surviving hen, under artificial light, started laying at 35 weeks of age and laid 14 eggs in the first month of laying. Seven of these eggs were incubated at Davis, and all were found to be fertile. Only one of these eggs hatched; however, the low

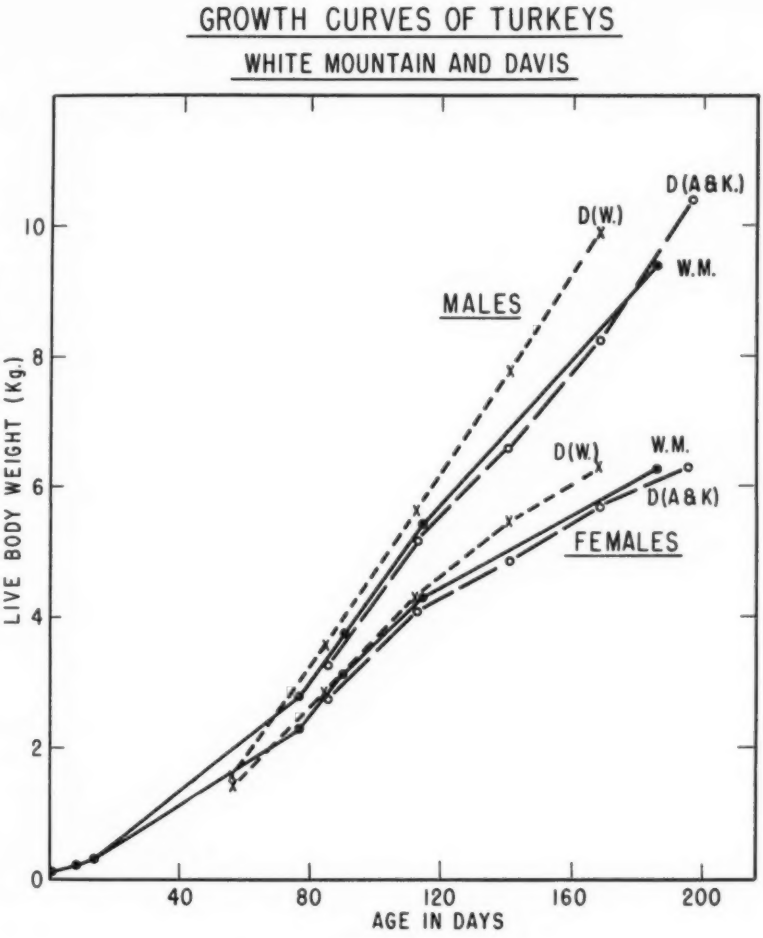

FIGURE 1

GROWTH CURVES OF TURKEYS AT WHITE MOUNTAIN AND AT DAVIS

The weights of the White Mountain birds at and prior to 76 days represent 7 ♂ and 9 ♀. The 90 and 114 day weights represent 5 ♂ and 8 ♀, and the 186 day weights represent 2 ♂ and 1 ♀. The curve marked D (A & K) is taken from Asmundson and Kratzer's Bulletin (1) and represents standard growth performance for similar stock at sea level. The curve marked D (W) represents a very large group of turkeys raised at Davis, over the same period as the White Mountain birds.

hatchability was considered due to damage in transit (via parcel post) rather than non-viability of the embryo.

The mean relative organ weights (i.e., gms. organ per 100 gms. body weight) were calculated for the high and low altitude groups. These data are compared, with White Mountain birds, as % of Davis-raised birds, in table 2. The relative organ weights of the Davis con-

TABLE 2
COMPARISON OF MEAN RELATIVE WEIGHTS OF ORGANS FROM WHITE MOUNTAIN AND DAVIS TURKEYS

(White Mountain Birds as % of Davis Birds)	11-12 Weeks	16-18 Weeks
No. of Birds	3:4	2:2
Heart	147*	110
Lung	85	70
Kidney	100	107
Spleen	73	117
Liver	110	110
Pancreas	125	100
Proventriculus	73	83
Gizzard	80	105
Adrenals	—	100

*Statistically Significant at the 5% level of random probability.

trols are quite similar to data of Marsden (8) and Latimer and Rosenbaum (7) for mature turkeys.

The only organ which showed a statistically significant weight difference in the high and low altitude groups, was the heart. At 11-12 weeks the heart weight of the White Mountain birds was 50% greater; but in older birds, 16-18 weeks, it was only 10% greater than Davis controls. Van Liere (13) has reported an average increased heart size (55%) in mature guinea pigs exposed to simulated high altitude (14-18,000 feet). One animal which received the longest treatment (105 days) "showed signs of atrophy but gave evidence of a previous hypertrophy." These observations suggest that some slower developing, perhaps cellular, adaptation process replaces an early accomodative increased heart output which causes an increased heart size. It is interesting to note Van Liere's statement (14) that adapted people at high altitude have normal heart rates and blood pressures.

The tremendous increase in relative spleen size (five-fold) observed by Van Liere (13) in his guinea pigs after exposure to simulated high altitude was not observed in the turkeys described herein.

This non-reactivity of the spleen may reflect a different structure and function of this organ in the bird. In the domestic fowl the spleen does not have the extensive trabeculae nor the muscle fibers which are found in the mammalian spleen (2). Sturkie (12) has tested the ability of the hen's spleen to store erythrocytes and, following certain stimuli, discharge them into the circulation, and has found no evidence of such a "reservoir" function.

The White Mountain turkeys showed a decreased relative lung weight (15 and 30%). This is quite in contrast to Hurtado's observations (6) on man in the Andes, from which he concluded that increased lung capacity, distension of alveoli, and engorgement of lung capillaries were major factors in adaptation to high altitude. The anatomy of the avian lung and respiratory system generally, as with the spleen, is quite different from that of the mammal (2). Resulting functional differences in the lungs of these two classes may account for their different growth reactions to high altitude.

Discussion

In evaluating the results presented here, it is desirable to evaluate the degree of hypoxic stress to which the experimental birds were subjected. The partial pressure of air in the bird's lung, 421 mm. Hg., can be calculated from the mean barometric pressure, 516 mm. Hg., by subtracting the water vapor pressure, 55 mm. Hg., and the CO_2 tension, 40 mm. Hg. The oxygen tension in the lungs of these birds would be 88 mm. Hg. Similar calculations indicate that the lung gas of sea level birds would have an oxygen partial pressure of 139 mm. Hg. Thus, at the 10,500 feet elevation, the lung oxygen has only 63% of the sea level concentration.

The effect of this reduced oxygen tension on the oxygenation of the bird's blood can be approximated from oxygen dissociation curves of the blood of other birds (5, figure 2). From these data it would appear as if the blood of the White Mountain turkeys was only 82-90% saturated with oxygen. The observations on behavior and organ growth indicate that this degree of hypoxia was physiologically significant. By comparison, an 84% oxygenation of hemoglobin in unadapted man leads to an appreciable handicap in performance.

The survival, normal growth and attainment of sexual maturity of the White Mountain turkeys indicate that they were successful in adapting to this degree of hypoxic stress. This apparent normality

would also indicate that the rations, which are based on "sea level" requirements for specific nutrients, are adequate in supporting growth and development up to 10,500 feet altitude. It has been suggested that the requirements of some vitamins, particularly thiamin and ascorbic acid (not considered a dietary requirement for birds), may be increased at high altitude (9). Since the thiamin content of the rations fed is very much larger than the recommended allowance, a large increase in the requirement could occur and be undetected. The nicotinic acid content of these rations, however, is only slightly above the recommended allowance, and the adequacy of the ration indicates that the high altitude induced no appreciable increase in its requirement. The single case of perosis which was observed is not considered indicative of a nutritional disturbance induced by high altitude, since this disease is encountered with this frequency (i.e., 3%) on these rations at sea level.

Summary and Conclusions

Day old broad-breasted bronze turkey poults were brooded and subsequently reared at an altitude of 10,500 feet. The rate of growth and attainment of sexual maturity was equivalent to that observed with similar stock at sea level. Anatomic studies indicated that a relative increase in heart size was the principal developmental change. The increases in lung and spleen size observed in mammals were not found in these birds.

Acknowledgment

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References

1. Asmundson, V. S., & Kratzer, H. H. 1951. Turkey production in California. Calif. Agric. Ext. Sta. Bull., No. 110. Pp. 60.
2. Bradley, O. C., & Grahame, T. 1950. The structure of the fowl. J. B. Lippincott, Philadelphia. Third edition. Pp. 128.

3. COATES, M. E., DICKINSON, C. D., HARRISON, G. F., KON, S. K., CUMMINS, S. H., & CUTHBERTSON, W. F. J. 1951. Mode of action of antibiotics in stimulating chick growth. *Nature,* **168**, 332.

4. COOK, S. F., & PACE, N. 1952. White Mountain High Altitude Research Station. *Science,* **116**, 697-700.

5. HALL, F. G., DILL, D. B., & BARRON, E. S. G. 1936. Comparative physiology at high altitude. *J. Cell. Comp. Physiol.,* **8**, 301-13.

6. HURTADO, A. 1932. Respiratory adaptation in the Indian Natives of the Peruvian Andes. Studies in high altitude. *Am. J. Phys. Anthropol.,* **17**, 137-65.

7. LATIMER, H. B., & ROSENBAUM, J. A. 1926-27. A quantitative study of the anatomy of the turkey hen. *Anat. Rec.,* **34**, 15-23.

8. MARSDEN, S. J. 1940. Weights and measurements of parts and organs of turkeys. *Poult. Sci.,* **19**, 23-28.

9. MITCHELL, H. H., & EDMAN, E. 1951. Nutrition and climatic stress. C. C. Thomas, Springfield. Pp. 234.

10. MOORE, C. R., & PRICE, D. 1948. Study at high altitude of reproduction, growth, sexual maturity, and organ weights. *J. Exp. Zool.,* **108**, 171-216.

11. STICKNEY, J. C., & VAN LIERE, E. J. 1953. Acclimatization to low oxygen tension. *Physiol. Rev.,* **33**, 13-34.

12. STURKIE, P. D. 1943. The reputed reservoir function of the spleen of the domestic fowl. *Am. J. Physiol.,* **138**, 596-602.

13. VAN LIERE, E. J. 1936. The effect of prolonged anoxemia on the heart and spleen in the mammal. *Am. J. Physiol.,* **116**, 290-4.

14. ———. 1942. Anoxia, its effect on the body. Univ. Chicago Press, Chicago. Pp. 269.

Growth, 1954, **18**, 37-51.

A SPECIFIC TYPE OF MICROORGANISM ISOLATED FROM ANIMAL AND HUMAN CANCER:[1] BACTERIOLOGY OF THE ORGANISM

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A specific type of microorganism has been observed, isolated, and cultivated consistently from every one of hundreds of specimens obtained from both animal and human cancer of various types over a five year period. It has a polymorphic developmental pattern which a number of others (1-9) in various parts of the world have observed wholly or in part. Our chief contributions to the bacteriologic study of this organism are (a) the recognition of its potential acid-fast properties by Wuerthele-Caspe (10-13) and (b) the demonstration by Alexander-Jackson (14-16) of the similarity of its developmental morphology to that of the mycobacteria of tuberculosis and leprosy which she had studied closely for more than fifteen years. These contributions towards an understanding of this pleomorphic mycobacterium-like organism which we have tentatively called *Mycobacterium tumefaciens*, were made possible by utilizing easily repeated stain technics and methods of cultivation which have not heretofore been applied in cancer research.

The outstanding characteristics of the organism apply to similar acid-fast microorganisms which we have isolated from various other proliferative and degenerative diseases of obscure etiology, including Hodgkin's disease, scleroderma, sarcoid, arthritis, and Wilson's disease (hepatoventricular degeneration). An experiment was carried out with blood from scleroderma patients involving the intraperitoneal inoculation of 30 white mice and 30 controls. During the last three years 621 other animals, including mice, guinea pigs, and chickens, were inoculated with pure cancer cultures. A number of chick em-

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bryos were also infected by inoculation of cultures in the chorio-allantoic membrane. The invasive, slow-working pathogenicity and type of pathology produced by the cultures from scleroderma* and the other afflictions mentioned above, suggest that this type of micro-organism may play a major role in a variety of chronic diseases (26).

The following characteristics are important in the identification of these microorganisms:

(1) Acid-fast staining properties—especially in tissues.
(2) Ability of the smaller forms to enter the cytoplasm and nucleus of host cells.
(3) Filterability of the smallest forms through Seitz or fritted glass filters.
(4) Large cyst forms within which develop acid-fast granules and rods.
(5) Failure to grow on ordinary media, and scant growth of primary isolates, which in from five days to two weeks form a granular mat at the bottom of liquid media, and on staining and in hanging drops reveal many symplasms. Cf. Fig. 1.
(6) Development at times in necrotic tissue, or in special media, of highly motile rods which form a pellicle that sinks readily to the bottom of the tube or flask. (5), (34), (35).
(7) Ability of the motile rods, when once established, to grow on ordinary media, and even at room temperature.
(8) Resistance of motile rods to (a) temperature—90 degrees Centigrade for 14 minutes—and (b) penicillin—0.1 ml of culture grew out in the presence of 1,000 units of penicillin per ml.
(9) Ability of Seitz filtrates from motile rod cultures to redevelop the rods after some weeks of incubation.
(10) Pathogenicity of both motile and non-motile cultures for animals. Death occurred in 24 hours when white mice received 0.2 ml and guinea pigs received 0.5 ml intraperitoneally of motile rods. Survivors develop a chronic systemic disease with granulomatous lesions, giving rise at times, in the case of the cancer organism, to neoplasms.
(11) Gram stain variability.
(12) Presence in motile rods of colorless areas similar to those observed by some in rods and filaments of tubercle bacilli. Cf. references (19), (20), (21), and Fig. 3.

*Confirmed, 1953, at Pasteur Inst., Brussels (32).

(13) Development in older cultures of mycelium-like filaments with rudimentary branching and budding.
(14) Fluorescence as shown by Gerlach (7) and Diller*.

Media

The principal media employed over a period of five years include Difco's brain-heart broth with and without glycerin, Dubos medium, Alexander-Jackson's modification of von Szabocky's glycerol lung broth, dextrose blood agar, Alexander-Jackson's adaptation of Bushnell's poi agar, Petragnani, Lowenstein-Jensen, Dorset egg media, and Wuerthele-Caspe's chick embryo agar. The embryonated egg was utilized in early work, and in the first successful efforts to induce motile rods to develop in non-motile cultures isolated from patients' blood. The living chick embryo proved an excellent medium. However, since some embryonated eggs contained mycobacterial forms, this medium is not one of choice for work involving mycobacteria or other acid-fast organisms. Growth of primary cultures on Sabouraud's medium was scant or negative. Our best results have been obtained with Alexander-Jackson's broth, and recently, with Wuerthele-Caspe's autoclaved chick embryo agar. The method of preparation of these two media is given below.

Alexander-Jackson's sensitive peptone broth:

Ingredients:

water (distilled)	2,000 ml
beef lung, cut up	2 pounds
peptones	20 grams; 5 grams each of (a) myosate, (b) gelysate, (c) trypticase, (d) phytone (obtained from Baltimore Biological Laboratory, Inc., 1640 Gorsuch Ave., Baltimore 18, Md.)
glucose	10 grams
glycerol	30 ml

Boil the beef lung and water for 30 minutes. Filter through cotton or very coarse paper into a flask containing the other ingredients, and heat to dissolve. This crude lung broth can be autoclaved and stored in the icebox, and clarified subsequently. Autoclaving for a second time does not seem to produce any adverse effects.

*Personal communication.

Clarification:

A 1- to 2-mm layer of infusorial earth (Standard Filter Cel of Johns-Manville Co.) is deposited on a No. 42 Whatman paper disc by laying the disc on a Buchner funnel, applying suction, and then carefully pouring on about 500 ml of a 5 per cent suspension of Filter Cel. After the deposition of the layer, when the water goes through clear, the suction flask is well rinsed out. The hot medium can now be filtered through the prepared disc into the flask.

The medium should be filtered a second time through a Buchner-type funnel with a fine fritted glass disc, or else passed once more through the same Filter Cel.

pH Adjustment:

The pH of the medium should be adjusted to 7.4 with sodium hydroxide. The medium is then tubed into screw-top glass tubes 150 x 25 mm (Kimble Glass Co., Toledo, Ohio). The tops of the tubes are not screwed tightly until after autoclaving. Autoclave for 15 minutes at 15 pounds pressure. Place about 5 ml of medium into each tube; or place 50 ml in a 250-ml Erlenmeyer flask for primary isolates. Close the flasks with cotton plugs held in a single layer of gauze, and protect the plug by a paper drinking cup or cone.

The peptones included in the above broth were studied individually. Myosate, a pancreatic hydrolysate of heart muscle, favored small virus-like and coccoidal forms. Gelysate, a gelatin hydrolysate, appeared to favor slender acid-fast rods and non-acid-fast rods containing acid-fast granules. Phytone, a papaic digest of soya meal (suggested by Dr. George A. Clark), and trypticase, a pancreatic digest of casein, both favored the readily growing motile rods. A combination of all four peptones provides a medium which allows a wide variety of forms to develop, and makes it easier to recognize the presence of the organism in primary isolations. Von Szabocky used Witte's peptone. Other peptones were tried which supported growth of isolates: Difco's bactopeptone, and Armour's peptone. A Merck peptone was used by von Brehmer, and Glover stated that certain "sensitive" batches of Fairchild's peptone were necessary in order to produce motile rods.

Wuerthele-Caspe's Chick Embryo Agar

The contents of 8-15 day old embryonated hens' eggs are ground up in a Waring blendor, mixed with 1.5 per cent melted

agar, tubed in screw-top glass tubes, preferably large ones, slanted, and autoclaved at 15 pounds for 20 minutes in the slanted position.

Isolation of Cultures

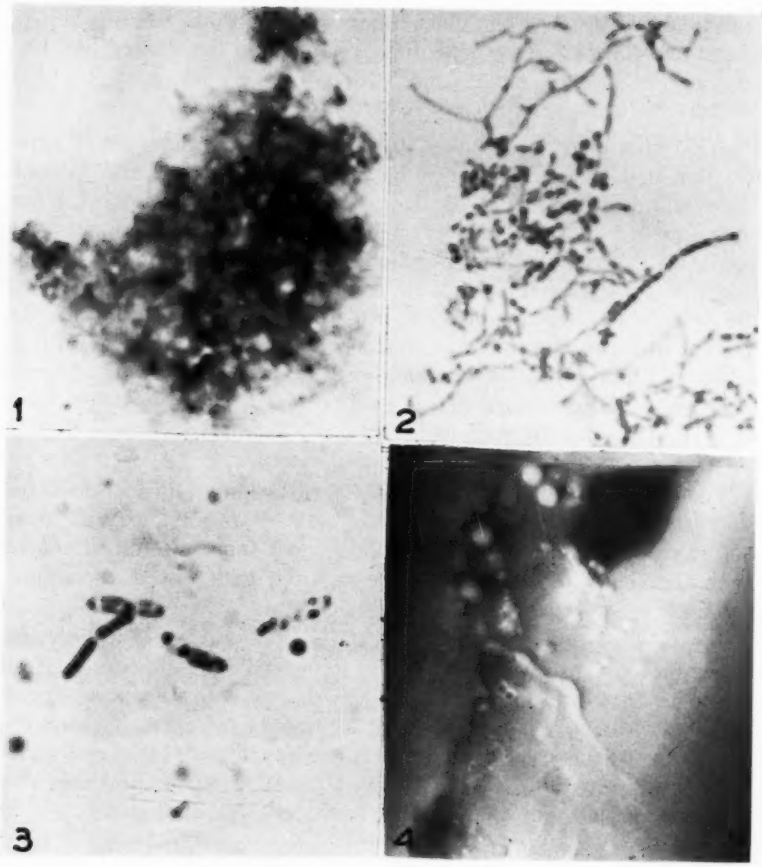
Blood specimens were taken by sterile hypodermic syringe after the skin had been well rubbed with tincture of iodine and alcohol. Upon withdrawing the needle from the vein, a few drops of blood were expelled to wash off any chance contaminant adhering to the needle as it passed through the skin; or else after removing the needle from the syringe, a few drops of blood were expelled before placing the balance into sterile tubes, or into tubes or flasks of broth. The amount of blood cultured was from 0.2 to 0.5 ml in 5 ml of broth in a 150 x 25 mm screw-top tube, or 2 to 5 ml of blood in 50 ml of broth in a 250-ml Erlenmeyer flask. It is important to have a shallow layer of liquid medium for sufficient aeration. Becton-Dickenson vacuum bottles were also employed in obtaining blood.

Tumors were usually cut up with sterilized instruments, extracted with normal saline, and the extract passed through a Seitz filter before cultures were made. Sometimes small snips carefully removed from the center of the tumor were cultured and proved uncontaminated by other organisms.

After incubation at 37°C for 10 days to 2 weeks, a generous amount of the primary culture was transferred by pipette to fresh medium. In 4 or 5 days granular growth was seen. Frequent transfer of at least 0.5 ml inocula and alternation of media helped the development of early cultures. Sterilized lumps of bone and plant charcoal appeared to have a slight stimulating effect, probably due to traces of salts and minerals.

Smears and unstained hanging drops were made from time to time. Agglutination tests were made in hanging drops by adding droplets of the culture to droplets of various sera. Any pellicle appearing on the surface of the culture was examined for the presence of motile forms, and then transferred. If the organisms showed acid-fast forms, they were tested for pathogenicity. In the case of our motile cultures, inocula of 0.2 - 4 ml into white mice and 0.5 ml into young guinea pigs proved fatal within 24 hours.

Smears were made on clean, new glass slides, and labeled with diamond pencils. Wax pencils were avoided because even a trace of wax



FIGURES 1-4

VARIOUS FORMS OF THE MICROORGANISM ISOLATED FROM HUMAN CANCER:

FIGURE 1. Zoogleal Form in Primary Blood Culture. 2500X. Grown on Alexander-Jackson's Sensitive Peptone Broth.

FIGURE 2. Coccoidal Forms and Filaments with Rudimentary Branching. 2500X. Dextrose Blood Agar.

FIGURE 3. Motile Rod Forms Showing Some Rods Breaking Up into Granules; the Smallest Ones at the Extreme Right Are Acid-fast. 2500X. Alexander-Jackson's Sensitive Peptone Broth.

FIGURE 4. Colonies of S Type on Dorset Egg Slant; Large Burnt-orange Pigmented Colony at the Right.

may be mistaken for granule forms of mycobacteria. Sterile capillary pipettes or slender glass tubing were useful for drawing up small amounts of granular or lacy growth directly above the red blood cells at the bottom of primary culture tubes or flasks. After allowing the films to dry in air, they were flamed on the underside rapidly 4 or 5 times.

Control blood specimens were obtained from blood bank donors and from other sources. None of the 88 blood bank donor cultures showed the organism. Blood cultures obtained from 12 healthy tuberculin-negative young nurses were also apparently free of the organism. However, we have found carriers of this type of organism in "normal" controls taken at random. A blind test was run on 25 blood cultures, 5 of which were cancerous, and on 20 applicants for jobs, in apparent good health. The cancer bloods were all positive, and 10 of the 20 control bloods also showed forms of this type of organism. Our study of bloods from controls is not sufficient to be of statistical significance, but our findings agree generally with earlier work by Glover and his associates* who found a large percentage of carriers—approximately 20 per cent of normals, and 40 per cent of an indigent elderly group. This does not seem strange when one considers the fact that according to vital statistics of recent date, one person in five eventually dies of cancer. It should also be remembered that healthy persons may carry other pathogens such as diphtheria bacilli, pneumococci, streptococci, tubercle and typhoid bacilli. Mice receiving the Bittner milk factor do not develop mammary carcinoma until middle age. It seems significant that we have obtained this microorganism in 100 per cent of cultures made by us from hundreds of human and animal neoplasms, and that the organisms apparently are identical to those obtained from cancer patients by workers in various other parts of the world, several of whom have visited our laboratory or have sent us their cultures.

Staining Methods

The staining technic which we have found especially useful is a triple-stain modification of the usual Ziehl-Neelsen method employed for staining acid-fast organisms. It was devised by Alexander-Jackson (14) to demonstrate non-acid-fast and non-bacillary forms of *Myco-*

*Personal communication.

bacterium tuberculosis which are not revealed by ordinary Ziehl-Neelson counterstains. When this differential stain was applied by Wuerthele-Caspe to smears from neoplastic tissues, striking mycobacterial forms were revealed. The triple stain also made evident minute acid-fast bodies not only within the cytoplasm, but even within the nucleus of cancer cells. Similar virus-like bodies were found in tumor filtrates and in primary blood cultures from cancer patients. These tiny elementary bodies, many of which are surrounded by a colorless area or capsule, and other larger forms, both acid-fast and non-acid-fast, have been completely overlooked or otherwise interpreted by most cancer workers. Stained sections of methylcholanthrene and fuel-oil induced tumors of the rat and rabbit, respectively, were obtained through the kindness of Dr. Margaret Lewis of the Wistar Institute, Philadelphia, and Dr. K. Sugiura of Sloan-Kettering Institute, Memorial Center, New York. These stained sections were decolorized in acid-alcohol, and after restaining with the Ziehl stain, bacterial forms showed up which appeared identical with those shown by the same stain on smears and cultures from naturally occurring cancers.

In applying the Alexander-Jackson triple-stain technic, details of which were published in *Science* (14) (1944), the Loeffler's methylene blue counterstain is made even more penetrating by the addition of a few drops of normal sodium hydroxide to each slide while it is covered with the blue dye. When studying a pure culture, one may then simply wash the slide, blot with clean bibulous paper, and examine. However, in smears from mixed cultures, tissue or body fluids, the excess methylene blue of the background is bleached out by freshly prepared dilute sodium hydrosulfite solution. Non-acid-fast mycobacterial forms resist this weak bleach, whereas all other non-acid-fast organisms, tissue cells, or background material are decolorized and take up a third dye—an acid green and acid yellow mixture. When washing out the unfixed carbol-fuchsin from both the leprosy and the cancer specimens, it has been found advisable to use 1 per cent HCl in 70 per cent alcohol for a few seconds or for as little time as is needed for proper differentiation, as these organisms appear to be much more alcohol-sensitive than the tubercle bacillus.

When using the ordinary Ziehl-Neelsen stain, we have found Victoria blue, as recommended by Gerlach (7), an excellent dye, as it is a clear flat blue devoid of the variations in tone often found in

methylene blue. Other stains employed at various times were Feulgen's, Fite's, Macciavello's, and the Hotchkiss-McManus stain. Sections were routinely stained by haematoxylin and eosin, and the regular Ziehl-Neelsen technic. The acid-fast bodies are Feulgen-positive, suggesting that whatever function (20, 27, 28) they may perform, they contain desoxyribosenucleic acid.

Morphology of the Organism

In addition to the Seitz-filterable intracellular acid-fast bodies of 0.2 μ previously mentioned, still smaller submicroscopic bodies ranging in size from 20 to 70 millimicrons were revealed in electron microscope photographs of our cultures taken by Hillier (11). These bodies are of viral dimensions. Larger coccoidal forms, often connected by slender filaments, range in size from 1.5 μ to cyst-like ring forms 3 to 4 μ in diameter. Still larger globi reminiscent of leprosy are found. Globoid forms suggest budding forms of fungi. The larger cyst forms in tissue, if not differentially stained, can be confused with cell nuclei of the host.

When cultivated on our media, the predominating form found in primary blood cultures from cancer patients and patients with the proliferative diseases we have studied* is a pleuropneumonia-like zoogleal matrix or symplasm, in which are embedded bodies of various sizes, some acid-fast, others non-acid-fast. This form has been described in detail by Gerlach (7). In patients with apparent resistance or who are undergoing treatment, round body forms are prominent. The coccoidal forms are sometimes difficult to distinguish from common cocci. However, they do not remain in the coccoidal phase in a favorable sensitive medium, but evolve either directly into rods by lengthening out, or else undergo a more complicated developmental process. This consists in enlargement into cyst-like ring forms. Small oscillating granules appear within the cyst, or bud from

*Brown and his co-workers (30) state: "It might be logical to suggest that there may be a more fundamental antigen (than that of the streptococcus) as a common denominator in all collagenous diseases."

"If a common antigen does exist in these diseases it must meet certain criteria. The persistence of the antigen in the affected areas might suggest that it is a living agent invisible in tissues. This agent should be capable of passing cell membranes and residing intracellularly in greatest concentration at the affected areas where the signs of clinical activity are most marked. It should be of low pathogenicity, and behave more as a parasite than as a virulent agent. Organisms of the pleuropneumonia group (L organisms) would satisfy these criteria."

its rim. In the very much larger globi, the rims break up into filaments and rod-shaped segments. Some of the granules within also elongate into rods. The granules, rods, and rims are often acid-fast on stained films. In old cultures, rods or filaments sometimes appear to coil up into rings, suggesting that under some conditions cysts can arise in this fashion.

In a culture developing in broth, cysts may become pear-shaped and discharge their inner contents through a short "neck". Grigoraki (29) has described such a phenomenon in some of his cultures of *M. tuberculosis*. The free granules, many of them in pairs, lengthen out into slender acid-fast rods, often with pointed ends. Sometimes needle-like acid-fast rods or filaments extending from a group of granules are star-like or have a sea-urchin aspect. Similar forms have been observed constantly in unstained hanging drops. Many of these rods are highly refractile and in tissues are sometimes surrounded by a substance possibly secreted either by them or by the host. They have an active Brownian motion.

The slender rods enlarge, and at this point the majority of them may develop true motility (34) by virtue of flagella, their motility varying from a lazy gliding and twisting, to intense activity. Motility, however, is not a permanent characteristic. In cultures allowed to age, even at room temperature, the rods lengthen out into motionless filaments which break up into round bodies. At times granules appear to bud out from the rods. Colorless areas which fail to take any of the stains used are also seen within some of the rods. Such non-staining areas have also been described for the tubercle bacillus by Coppen-Jones (19), Knaysi, Hillier and Fabricant (20), and by Brieger (21). With our stain technic one can occasionally observe in the center of the unstained area one or a pair of very minute bodies, which with their colorless surrounding vacuole or capsule resemble inclusion forms found by Wuerthele-Caspe in tumor tissue.

Filtrates of Seitz-passed motile rod cultures, in which only tiny virus-like bodies could be found, redevelop motile rods after a month of incubation at 37°C in our sensitive broth. This fact would seem to preclude their belonging to the true viruses or that they are viruses carried along by the rods. It is of interest to mention that Brieger (19) has observed virus-like forms in infective tuberculous tissue extracts, and Alexander-Jackson's study of isolates from the blood of leprosy patients strongly indicates the existence of virus-like phases

of *Mycobacterium leprae*. Further close study of single-cell isolates in microdroplets is necessary to prove beyond doubt that these amazingly plastic fungus-like bacteria actually generate virus-like forms which are part of their life history.

It may well be that the known cancer "viruses" such as the Rous sarcoma virus, the mouse leukemia agent of Gross (*22*, *23*) the Bittner milk factor concerned in mouse mammary carcinoma, and even Shope's papilloma virus of rabbits, actually represent filterable virus-like phases of pleomorphic bacteria. The results of our studies made from human and animal tumors, blood cultures inoculated into a variety of animals, and cultures grown from the Rous and Bittner agents, suggest that this may be true. Neoplasms have arisen in some of the various animals inoculated with pure cultures, notably in guinea pigs, which rarely develop spontaneous cancer. Koch's postulates have been fulfilled by the repeated re-isolation from infected animals of cultures of the inoculated organism.

Both motile and non-motile cultures isolated from cancerous blood and tumors undergo immediate clumping with loss of motility in the presence not only of specific sheep and rabbit antisera, but also in the presence of sera from animals immunized against tumor extracts, and in the presence of sera obtained from convalescent cancer patients. Moreover, preliminary protection experiments with rabbit antisera on culture-infected mice and leucotic chickens have given encouraging results. More extensive immunological experiments are now under way with sheep sera obtained from a small flock immunized with attenuated cultures isolated from cancer, scleroderma, arthritis, and Wilson's disease. It is of interest to note that cross reactions occur among the various strains in agglutination tests in hanging drops, and in skin tests with various antigens upon infected guinea pigs. None of the guinea pigs tested gave any reaction to tuberculin. The organism from Wilson's disease resembled that isolated from Hansen's disease and gave excellent Vi type of agglutination in serum from a leprosy patient. The guinea pig infected with this newly discovered pathogen of Wilson's disease gave a slight reaction to Carville lepromin. One of two leprotic guinea pigs also reacted to Carville lepromin. Guinea pigs infected with the organism isolated from cancer failed to react to lepromin. However, organisms from leprosy patients gave marked cross agglutination reaction in sera from animals inoculated with cancer strains and vice versa. Serum from a lep-

rotic patient agglutinated and immobilized motile rods obtained from blood of cancer patients. These results are not incongruous when one bears in mind that BCG is now being tried to immunize children against leprosy, and that red cells sensitized with tuberculin are being employed in a test with sera from Hansen's disease patients (24). Such facts point to common antigens among these various acid-fast organisms.

Organisms of plant cancer. It also seemed of interest to investigate the organism of plant cancer currently known as *Agrobacter tumefaciens,* using the same methods employed in working with isolates from human and animal cancers. Accordingly, strain A-6, a strain of *A. tumefaciens* virulent for plants, was obtained from Dr. A. J. Riker of the University of Wisconsin. This highly motile strain was placed in Alexander-Jackson broth and stained from time to time with the Ziehl-Neelsen and triple stains. Initially, the rods were non-acid-fast, but red-staining acid-fast bodies could be seen within a number of them when stained with the Ziehl-Neelsen method. After several weeks some of the bodies were noted lying free. Such a culture was inoculated intraperitoneally into six white mice. An equal number of control mice received broth alone. All the mice survived infection and were sacrificed three months later. The livers were pale, but no other abnormality was seen. Smears made from the organs showed large numbers of semi-acid-fast rods in the liver and spleen. Some of these were in cord-like arrangement, calling to mind cords of *Mycobacterium tuberculosis* described by Middlebrook (25). Here and there characteristic Y forms were seen but were acid-fast.

The organisms of plant cancer were immobilized and agglutinated by sera from sheep immunized against cultures derived from human cancer, and also by sera from sheep immunized against human tumor extract. Further experiments including biochemical studies are needed to determine whether or not *Agrobacter tumefaciens* is actually one of the mycobacteria.

Summary

A specific microorganism with a polymorphic developmental pattern has been isolated and cultivated consistently, in special media described, from every one of hundreds of specimens of blood or tumor tissue obtained from cancerous animals and human cancer patients over a five year period. Its various forms range in size from 20 μ to 4 μ. The organism has acid-fast as well as non-acid-fast forms,

which in cancerous tissue are especially well demonstrated by Alexander-Jackson's triple stain.

The organism was pathogenic for mice, guinea-pigs, and chick embryos. Its motile rod form in definite amounts killed the animals within 24 hours. The lesions of surviving animals were granulomatous, neoplastic, or showed intergradations. Seitz-filtered cultures, after prolonged incubation in the above-described media, redeveloped the larger forms, indicating that the filter-passing bodies are a stage of this polymorphic mycobacterium-like organism.

Animal and human cancer strains, and *Agrobacter tumefaciens* were immobilized and agglutinated by the sera of sheep and rabbits immunized against human tumor extract, and against cultures derived from human cancer.

References

1. Mori, N. 1932. "Hypothese sur la nature microbienne du cancer," *L'Hygiene sociale,* **101**, 2032.
2. von Brehmer, W. 1934. "Siphonospora polymorpha, n. sp. ein neuer Mikroorganismus des Blutes und seine Bezeihung zur Tumorgenese," *Med. Welt,* **8**, 1179-1185.
3. L'Esperance, E. S. 1931. "Studies in Hodgkin's Disease," *Ann. Surg.,* **93**, 162-168.
4. Nuzum, J. W. 1924. "Experimental Production of Metastasizing Carcinoma in Breast of Dog and Primary Epithelioma in Man by Repeated Inoculation of Micrococcus Isolated from Human Breast Cancer," *Surg. Gynec. Obst.,* **11**, 343-352.
5. Glover, T. J. 1930. "The Bacteriology of Cancer," *The Canada Lancet and Practitioner,* **74**, 92-111.
6. Clark, G. A., Engle, J. L., & Glover, T. J. 1944. "Report on the Metastasizing Tumors in Guinea Pigs and Albino Rats Following the Inoculation of a Microorganism Obtained from Human Cancer," unpublished.
7. Gerlach, F. 1948. "Krebs und obligater Pilsparasitismus," Wien, Urban und Schwartzenberg.
8. Villaquez, E. J. 1952. "Isolement de micro-organismes des tissues hyperplastiques et du sang des cancereux," *Concours Med.,* **74**, 661-664.
9. Diller, I. C. 1952. "Cytological Studies of the Relationships Between Microorganisms Isolated from Tumor Tissue and Normal and Malignant Cells of the Host," *Cancer Research,* **12**, 257; also Fourth Scientific Report, The Institute for Cancer Research, and The Lankenau Hospital Research Institute, Philadelphia, Pa.
10. Wuerthele-Caspé, V., & Allen, R. M. 1948. "Microorganisms Associated with Neoplasms," *N. Y. Microscopical Society Bulletin,* **2**, 2-31.
11. Wuerthele-Caspé, V., Alexander-Jackson, E., Anderson, J. A., Hillier, J., Allen, R. M., & Smith, L. W. 1950. "Cultural Properties and Pathogenicity of Certain Microorganisms Obtained from Various Proliferative and Neoplastic Diseases," *Amer. J. Med. Sciences,* **220**, 638-646.
12. Wuerthele-Caspé, V. 1949. "Mycobacterial Forms Observed in Tumors," *J. Am. Med. Women's Assoc.,* **4**, 135-141.
13. Wuerthele-Caspé, V., Alexander-Jackson, E., & Smith, L. W. 1953. "Some Aspects of the Microbiology of Cancer," *J. Am. Med. Women's Assoc.,* **8**, 7-12.

14. ALEXANDER-JACKSON, E. 1944. "Differential Triple Stain for Demonstrating and Studying Non-acid-fast Forms of the Tubercle Bacillus in Sputum, Tissue and Body Fluids," *Science,* **99**, 307-308.

15. ————. 1945. "Hitherto Undemonstrated Zoogleal Form of Mycobacterium Tuberculosis," *Ann. N. Y. Acad. Sciences,* **46**, 127-151.

16. ————. 1951. "Cultivation and Morphological Study of a Pleomorphic Organism from the Blood of Leprosy Patients," *International J. Leprosy,* **19**, 173-186. See also Proc. 6th Int. Cong. Leprosy, 1953 (in press).

17. LEVINE, M., & SCHOENLEIN, M. S. 1930. "A Compilation of Culture Media for the Cultivation of Microorganisms," p. 536. Baltimore: The Williams and Wilkins Co.

18. BUSHNELL, O. A., & ICHIRIU, E. T. 1950. "A Poi Agar Medium for the Isolation and Cultivation of Tubercle Bacilli," *Hawaii Med. J.,* **9**, 319-321.

19. COPPEN-JONES, A., in BARLARO, P. M. 1929. "Tratado de Tuberculosis," Tomo II, Libro I, pp. 14 and 46, Buenos Aires, A. Lopez.

20. KNAYSI, G., HILLIER, J., & FABRICANT, C. 1950. "Cytology of an Avian Strain of Mycobacterium Tuberculosis Studied with Electron and Light Microscopes," *J. Bact.,* **60**, 423-447.

21. BRIEGER, E. M., & GLAUERT, A. M. 1953. "A Study of the Reproduction of the Tubercle Bacillus," *Tubercle,* May, 128-138.

22. GROSS, L. 1951. "Pathogenic Properties and 'Vertical' Transmission of the Mouse Leukemia Agent," *Proc. Soc. Exper. Biol. and Med.,* **78**, 342-348.

23. ANDREWES, C. H. 1953. "Some Current Concepts of Viruses and Tumors," *The Journal-Lancet,* Minneapolis, **73**, No. 3, 87-92.

24. LEVINE, M. 1951. "Haemagglutination of Tuberculin Sensitized Sheep Cells in Hansen's Disease (Leprosy)," *Proc. Soc. Exper. Biol. and Med.,* **76**, 171-173.

25. MIDDLEBROOK, G., DUBOS, R. J., & PIERCE, C. 1947. "Virulence and Morphological Characteristics of Mammalian Tubercle Bacilli," *J. Exper. Med.,* **86**, 175-184.

26. FLEISHER, M. S. 1952. "Significance of Diphtheroid Microorganisms in Blood Cultures from Human Beings," *Amer. J. Med. Sciences,* **224**, 548-553.

27. MUDD, S., WINTERSCHEID, L. C., DELAMETER, E. D., & HENDERSON, H. J. 1951. "Evidence suggesting that the Granules of Mycobacteria are Mitochondria," *J. Bact.,* **62**, 459-475.

28. HOFFMAN, H. 1951. "The Cytochemistry of Bacterial Nuclear Structures," *J. Bact.,* **62**, 567.

29. GRIGORAKI, L. 1944. "Senescence et Evolution," Vol. I, Chap. 3. Paris: Le Francois Edit.

30. BROWN, T. M. *et al.* 1951. "A Study of the Antigen-Antibody Mechanism in Rheumatic Diseases," *Amer. J. Med. Sciences,* **221**, 618-625.

31. WUERTHELE-CASPÉ, V., BRODKIN, E., & MERMOD, C. 1947. "Etiology of Scleroderma, A Preliminary Clinical Report," *J. Med. Soc. New Jersey,* **44**, No. 7, 256.

32. DELMOTTE, N., & VAN DER MEIREN, L. 1953. "Recherches bacteriologiques et histologiques concernant la sclerodermie," *Dermatologica,* **107**, No. 3, 177-182. (Basel, New York).

33. WUERTHELE-CASPÉ, V., ALEXANDER-JACKSON, E., GREGORY, M., DILLER, I., & MANKOWSKI, Z. 1954. "An Intracellular Acid-Fast Microorganism Isolated from Two Cases of Hepatolenticular Degeneration" (Wilson's Disease), in press.

34. JENSEN, H. L. 1953. "The Genus Nocardia (or Proactinomyces) and Its Separation from other Actinomycetales with Some Reflections on the Phylogeny of the Actinomycetes," Symposium on Actinomycetales, VIth International Congress of Microbiology, Rome, pp. 69-88. (Supplemento ai Rendiconti del l'Instituto Superiore di Sanita).

35. CALMETTE, A. 1923. "Tubercle Bacillus Infection and Tuberculosis in Man and Animals," Williams and Wilkins Co., Baltimore. P. 14.

DIAGRAM OF THE VARIOUS FORMS OF THE POLYMORPHIC MICRO-ORGANISM CONSISTENTLY ISOLATED FROM THE BLOOD AND TUMORS OF CANCER PATIENTS AND ANIMALS.

1. Cysts or "spore-sacs" filled with acid-fast bodies. 2-4 microns.
2. Acid-fast bodies .prouting hair-like filaments.
3. Needle-like rods with pointed ends.
4. Larger rods, which may become motile.
5. Spore-like bodies.
6. Filaments and ring forms.
7. Minute acid-fast bodies .2μ.
8. Globoidal bodies .5μ to 2μ; sometimes refractile.
9. and 10. "Zoogleal" matrix forms or symplasms.

Growth, 1954, **18**, 53-69.

DEVELOPMENTAL PATTERNS IN THE BARROW GROUND SQUIRREL, *SPERMOPHILUS UNDULATUS BARROWENSIS*

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INTRODUCTION

The growth and development of young ground squirrels of the genus *Spermophilus* have been mentioned in the work of several authors. The papers of Johnson (3) and Wade (10) on *Spermophilus tridecemlineatus*, Shaw (9) on *Spermophilus columbianus*, Svihla (8) on *Spermophilus townsendii*, and Fitch (1) on *Spermophilus beecheyi* are all concerned, in some measure, with this problem. None of the authors named, however, have made a detailed study of the growth and development of ground squirrels from their natal day through the first year of adult life, although Johnson (3) has presented the most complete treatment of this subject to date. In the papers of Mayer (4, 5, 6, & 7) dealing with the Barrow ground squirrel, *Spermophilus undulatus barrowensis*, some mention is made of its breeding and growth. As so little about this animal has been previously published and since almost nothing is known of its development, this paper has been written to provide such information.

The Barrow ground squirrel (Figure 1) is found along the coastal tundra of Alaska north of the Brooks range, as indicated by Howell (2, p. 91) and Mayer (5, p. 49 & 6, p. 335). Its distribution over the tundra is not uniform but rather is limited to small areas of suitable habitat where the permafrost table is sufficiently low to allow the construction of burrow systems. Because of the unique ecological niche occupied by this animal and the severe climatic rigors to which it is subjected, it offers an unusual opportunity to study growth and development which of necessity must be accelerated to allow the young of the year to attain sufficient size and weight during the brief summer to enter hibernation in the fall.

FIGURE 1
Adult *Spermophilus undulatus barrowensis* in typical stance by burrow entrance. August, 1951. Teshekpuk Lake, Alaska.

In their habitat on the wet, flat, treeless, arctic tundra the ground squirrels are exposed to extreme cold during the months from October to May. Although certain rodents such as lemmings of the genera *Lemmus* and *Dicrostonyx* are active throughout the winter period, the ground squirrel resorts to hibernation to avoid the rigors of the environment. During a maximum activity period of five months adults must gain back not only weight lost during the previous winter's hibernation but also that lost in the demanding post-emergent breeding season. In three months of above-ground foraging the young of the year must attain sufficient size and weight to allow them to enter hibernation in the fall and to emerge in the spring in breeding condition.

Growth and Development in Adult Animals

Adults enter hibernation in September and emerge early in May of the following year. Fifteen adults weighed during May averaged 715.3 grams, varying in weight from 407.9 to 1018.6 grams. The five males averaged 920.9 grams (813.3-1018.6 grams), while the ten females averaged 612.5 grams (407.9-959 grams). During the post-emergent month the animals may live largely on stocks of food stored in the nest the previous fall, for the dead, dry surface vegetation of the previous year is frozen and covered with snow. During May breeding takes place, one instance of mating having been positively established as occurring on May 24th (Mayer 5, p. 52).

The total adult population of the study area during June was eighteen animals. The average weight for the population was 659.1 grams, varying from 504 grams to 810.7 grams. This represents a weight loss for the total population of 56.2 grams, or a decrease of 7.5% from the weight in May. Despite the fact that almost all the eleven females became pregnant by the first weeks of June and were post-partum and lactating by the latter part of the month, their weight average was 633.8 grams (504-801 grams), a gain of 21.3 grams or 3.5% over their May weight. The seven males, however, averaged 699 grams (569-810 grams), showing a loss in weight of 221.9 grams or 24% of their weight in May. During the breeding season the polygamous males range widely in their search for mates. This hyperactivity undoubtedly is related to their June weight loss. It is also possible that the breeding season increases the males' demand for food and causes them to exhaust their stocks of stored food more

quickly than do the females. With green surface vegetation not yet available, this lack of stored foods could be a major contributing factor to the males' loss of weight.

In July the ground is freed of snow and ice, and the dead surface vegetation of the previous year is exposed and replaced by abundant, fresh, green vegetation. Released from the pressures of the mating season, and with succulent new food readily available, sixteen adult animals in the study area in July averaged 779.3 grams (622.9-956.7 grams) in weight, an increase of 18% over the weight in June. The greatest gain was made by the five males of the population which averaged 870.7 grams (801.7-956.7 grams) or 24.5% over the June weight. The eleven females, which were still lactating during the first weeks of July, averaged 737.7 grams (622.9-839.5 grams) or 16.3% over the June weight.

The weight of the adult population of fifteen animals in August averaged 924.9 grams (671.9-1260 grams), an 18.6% increase over the July weight. The five males averaged 1027.4 grams (870-1260 grams), a 17.9% increase over the July weight, and the ten females averaged 873.6 grams (671.9-1143.5 grams), an 18.4% increase over the July weight. The greater increase for the females during August may be accounted for by the fact that they are now entirely free of caring for their young.

Only two adult animals were captured during September, indicative of the fact that most of the adults enter hibernation during August. The weight of these two averaged 933.6 grams (916.2-951 grams), a gain of but 8.7 grams or 0.9% over the average August weight. This slight gain is in marked contrast to the large weight gains shown in the months of July and August and indicates that the squirrels have already attained sufficient weight by August to permit them to enter hibernation without utilizing the month of September for foraging. That this is an advantageous adaptation is easily understood in view of the fact that by the 15th of September the first frosts of fall had already killed the green, summer vegetation of the study area. By the 22nd of September the first snows had already fallen, and by the 29th of September the lakes had started to freeze again, so that the situation in September as regards available vegetation resembled that of the previous June.

The total weight gain for the adult population from May emergence to September hibernation averaged 30.5%. Comparing the Sep-

tember pre-hibernation weight of 933.6 grams with the May emergent weight of 715.3 grams, it is evident that during the eight-month hibernation period the adults lost 218.3 grams, or 23.3% of their September weight. During hibernation the males lost 106.5 grams, or 10.3% of their August weight, and the females, 338.5 grams, or 35.5% of their September weight. This difference in weight loss is not clearly understood and can hardly be explained on the basis of the females' preparation for spring breeding as the males are likewise in preparation. Other reasons will have to be studied in order to explain this difference between the sexes.

The weight changes for adults are summarized in Figures 2, 3, and 4.

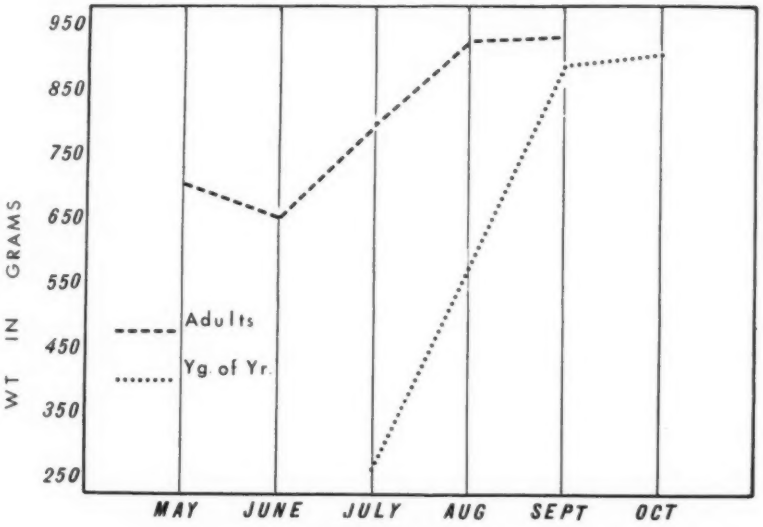


FIGURE 2
Comparison of weight changes of adults and young of the year at the Meade River study area.

Growth and Development of the Young

The young *Spermophilus undulatus* are born in June after a gestation period of 25 days. The number of young per litter varies from five to ten, the average of six litters being 7.8. The average birth weight of six litters ranged from 9.7 to 13.2 grams. In one litter of

five animals the individuals averaged 12 grams (11.1-12.8 grams), in another litter of eight they averaged 9.7 grams (8.1-11.5 grams), and in a litter of nine they averaged 10.8 grams (9-12.1 grams). In contrast to these figures Shaw (9, p. 109) reports the average birth

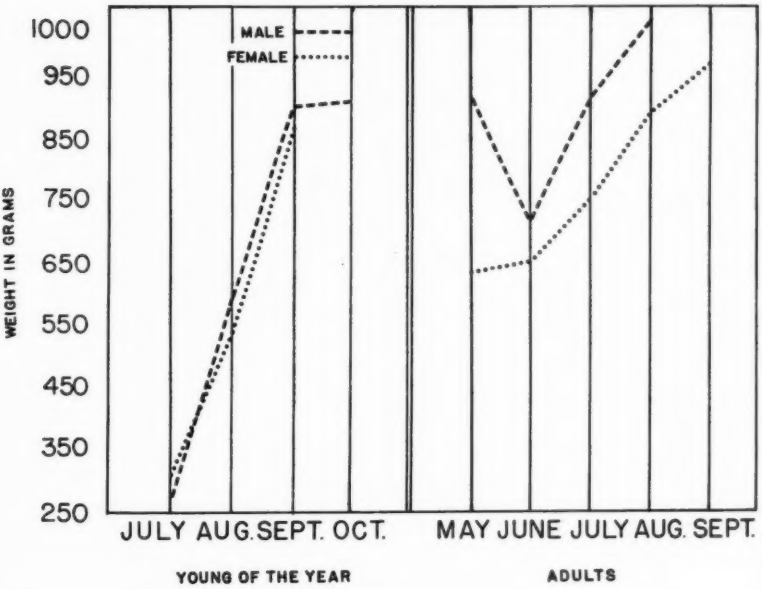


FIGURE 3
Weight changes of adults and young of the year shown in Figure 2 analyzed according to sex.

weight of individuals in a litter of *Spermophilus columbianus* to be 8.6 grams, after a gestation period of from 23 to 24 days. Wade (10, p. 272) reports a 27 to 28 day gestation period for *Spermophilus tridecemlineatus,* with an average weight of 2.6 grams for each of the newborn young. The average weight for newborn *Spermophilus townsendii,* as reported by Svihla (8), is 3.7 grams. *Spermophilus beecheyi* in California most closely resembles *Spermophilus undulatus* in adult size, and, as reported by Fitch (1, p. 578), the average weight of newborn young in six *S. beecheyi* litters was 8.9 grams. This consideration of comparative weights is indicative of the rapid development

of *S. undulatus* in the relatively short gestation period of 25 days. This rapid pre-natal growth is an obvious adaptive advantage in an animal which will have such a short post-natal growing season.

Although comparative size figures for young animals are not readily

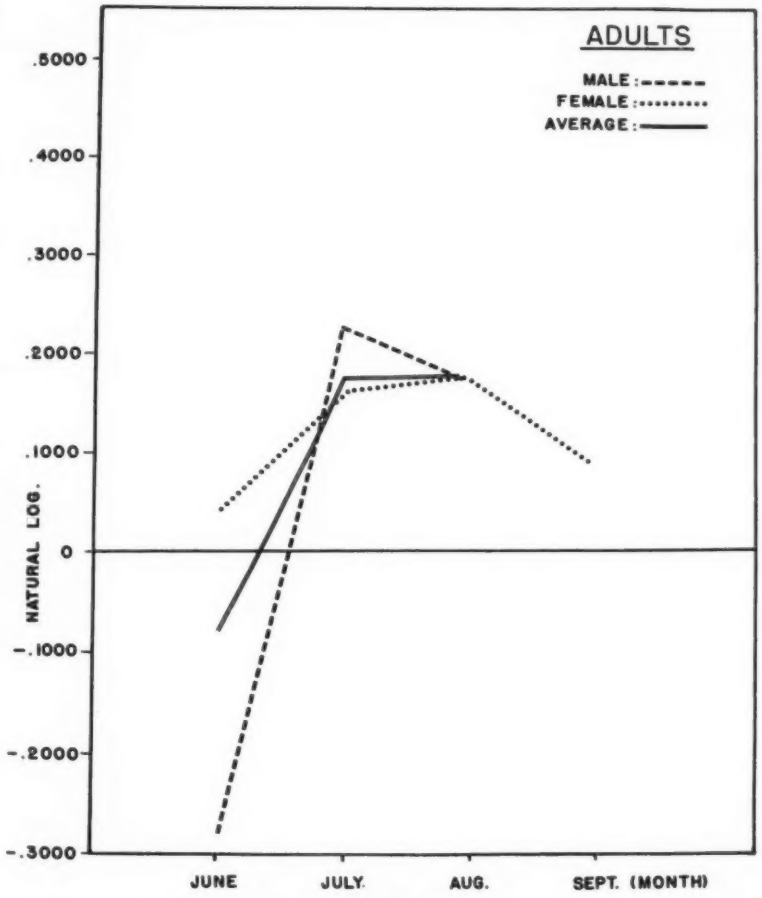
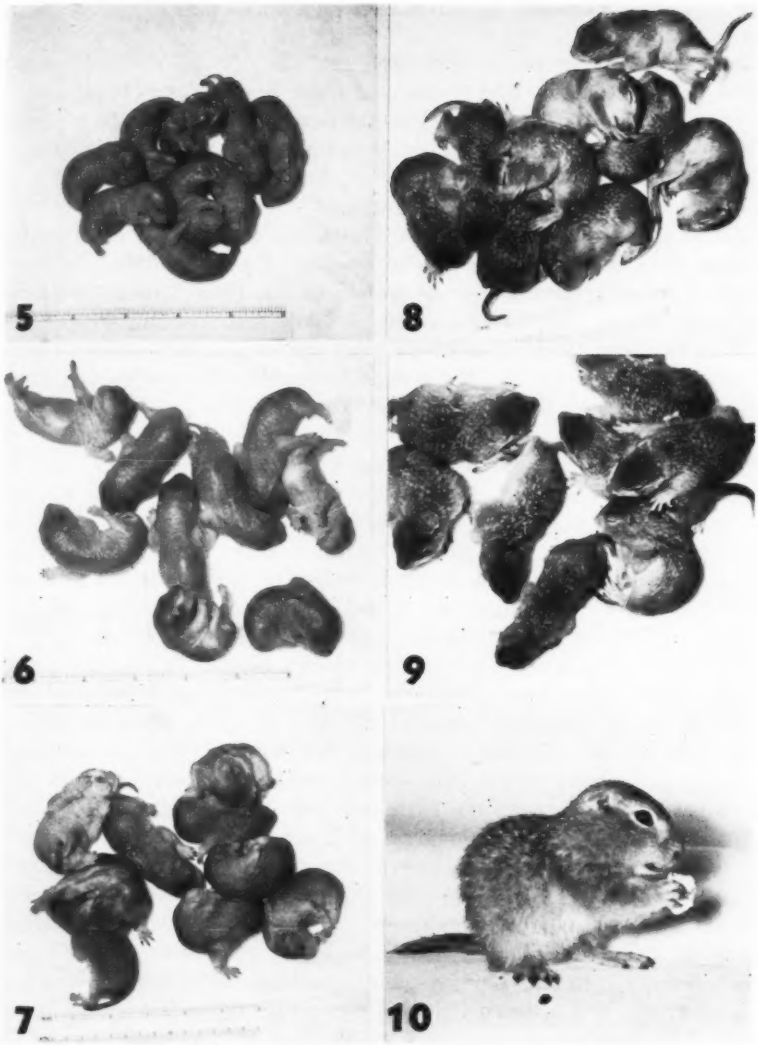


FIGURE 4
Weight changes of the adult population of the study area plotted as the natural log W_2 minus the natural log W_1, showing instantaneous growth rates for the months of surface activity.

available in the works of other authors, Svihla (8) presents some data on *S. townsendii,* indicating that the total length of newborn young averaged 52.6 mm., the tail length averaged 6.29 mm., and the hind foot had a mean length of 6.26 mm. The same measurements for *S. undulatus* newborn are 66.1 mm., 10.4 mm., and 8.3 mm. respectively. These figures, like those for weight, show the relatively large size of *S. undulatus* young when compared to other spermophiles.

At birth the young Barrow ground squirrels are blind, naked, red, fetal-like objects, with the pinnae scarcely noticeable and the pigmented eyeball showing clearly through the skin (Figure 5). They are capable of squeaky vocalization and can move slowly by wriggling the body and pulling themselves along with their fore-limbs, which are relatively well developed. By the second day after birth the beginning of hair growth is noticeable, accompanied by darkening of the dorsum. Eyelashes, vibrissae, and short head hairs are apparent at this time, and the hind-limbs are starting to play a more important role in locomotion. By the fourth day (Figure 6) pigmentation, following a cephalocaudal gradient, has extended further posteriorly and has darkened considerably. Additional pigmentation is noticed in the toes and on the tips of the ears. The umbilicus may still be apparent at this time. By the sixth day the vibrissae have reached a length of five millimeters, and both pigmentation and hair growth on other parts of the body have continued to develop. By the eighth day pigmentation has spread ventrally, and the beginnings of the adult dorsal spotting are noticeable. By the tenth day (Figure 7) short hairs cover the entire body, including the venter. While the chestnut color of the head is now apparent, the venter remains white. Pigment has invested the eyelid, and the external ear has reached a length of one millimeter. The voice is better developed and deeper sounds are substituted for the initial squeaks. By the 14th day a growl can be uttered. Continued development of the hair coat and more directed locomotion are noted throughout this period. By the 18th day (Figure 8) the young can produce the chatter so characteristic of the adult, and by the 20th day the eyes of most of the young have opened. By the 22d day (Figure 9) the young evert the papillae of the anal glands in the pattern so characteristic of the adult (Mayer 5, p. 339), scold, scratch, and behave like adults in a'most every way. They have begun to take nourishment from



FIGURES 5-10. STAGES IN THE DEVELOPMENT OF YOUNG *Spermophilus undulatus barrowensis.*

FIGURE 5. Litter of nine on natal day.
FIGURE 6. Litter of nine four days old.
FIGURE 7. Litter of nine ten days old.
FIGURE 8. Litter of nine eighteen days old.
FIGURE 9. Litter of nine twenty-two days old.
FIGURE 10. One young of litter of nine at twenty-eight days of age eating laboratory diet.

sources other than the mother, and can soon be maintained exclusively on a laboratory diet if removed from their mother (Figure 10). From this time on the developmental pattern is chiefly one of continued increase in size and weight, accompanied by a change in pelage from juvenile to sub-adult coat.

Data were collected on the growth of young animals both in the field and in the laboratory. For illustrative purposes a laboratory-raised litter of nine young can be considered as typical for the species. Figure 11 summarizes the growth of this litter through the first 44 days of life and shows a relatively slow and constant weight increase to about 20 days, at which point the weight had reached 109.9 grams. During this time the weight increments did not exceed 13.7 grams for any 48 hour period. Between the 20th and the 42d day, however, weight increased much more rapidly, going from 109.9 grams to 542 grams, with a maximum 48 hour increment of 60 grams. This 23 day period between the 20th and 42d days was the period of most rapid weight increase, the weight curve leveling off after the 42d day. The total gain during this time was 432 grams, or 53.2% of the average August adult weight of 924.9 grams. Thus by the 42d day of life the young ground squirrels had reached 58.6% of the weight attained by adults prior to entering hibernation.

Weight changes are again considered in Figure 12, in which the weight gains have been plotted as instantaneous growth rates of the natural log W_2 minus the natural log W_1. This interpretation of the data shows that the weight changes follow a bimodal pattern, the highest peak being between the second and the sixth day and the second peak being between the 24th and 28th days. Near the 20th day the plot shows a less rapid growth, indicative of the fact that the young have now reached a size at which weight increment can no longer be maintained at the previous rate by nursing alone. Additional food sources are apparently necessary if increase in weight is to be maintained at a high rate. When the eyes of the young open, between the 20th and 22d days, they can begin to utilize new sources of food, and it is believed that this fact accounts for the second spurt in weight increase.

Referring again to Figure 11, it can be seen that the total length of the young increased in a linear fashion from birth to the 42d day, when it reached a length of 336.1 millimeters, or 85.5% of the average adult length of 393.2 millimeters. The young animals had

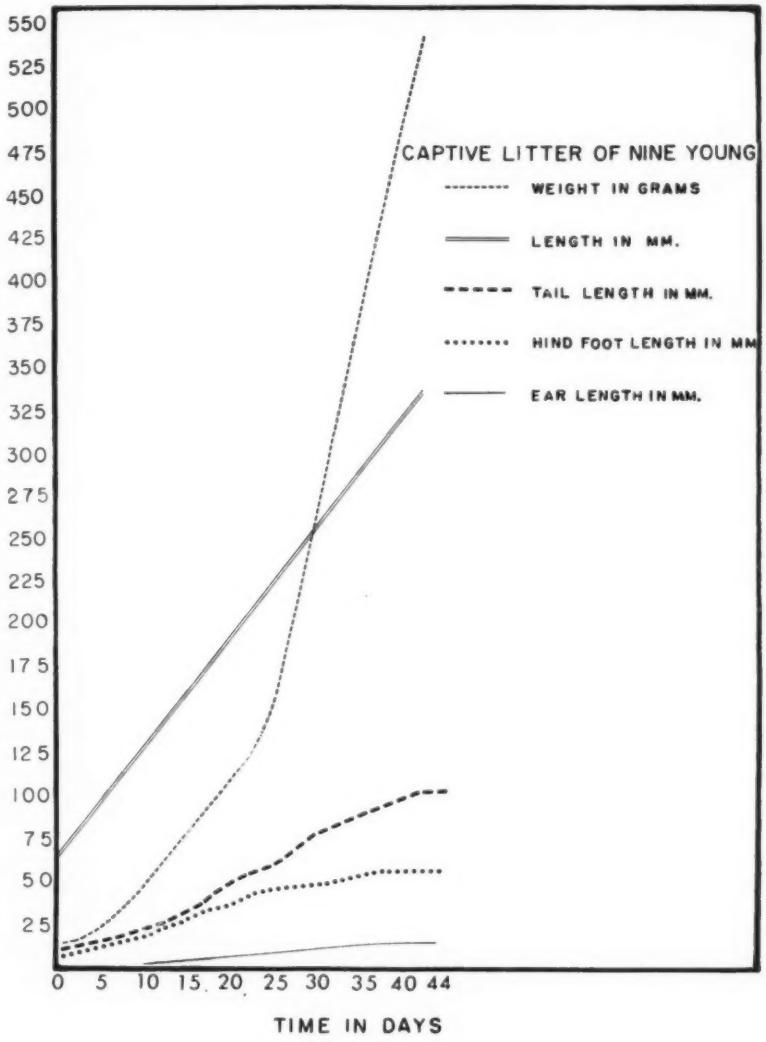


FIGURE 11
Growth patterns of litter of nine, illustrated in Figures 5 to 10, plotted as actual growth rates in days.

thus attained approximate adult length by the 42d day of life. In Figure 5 the instantaneous growth rate plot for total length shows peaks roughly similar to those for weight.

Figure 11 shows the plots of the growth rates for the tail length, hind foot length, and ear length in millimeters. These can be seen to be relatively typical growth curves. The tail reached an average length of 101.4 millimeters by the 42d day, or 88.6% of the average adult tail length of 114.4 millimeters. By the 38th day

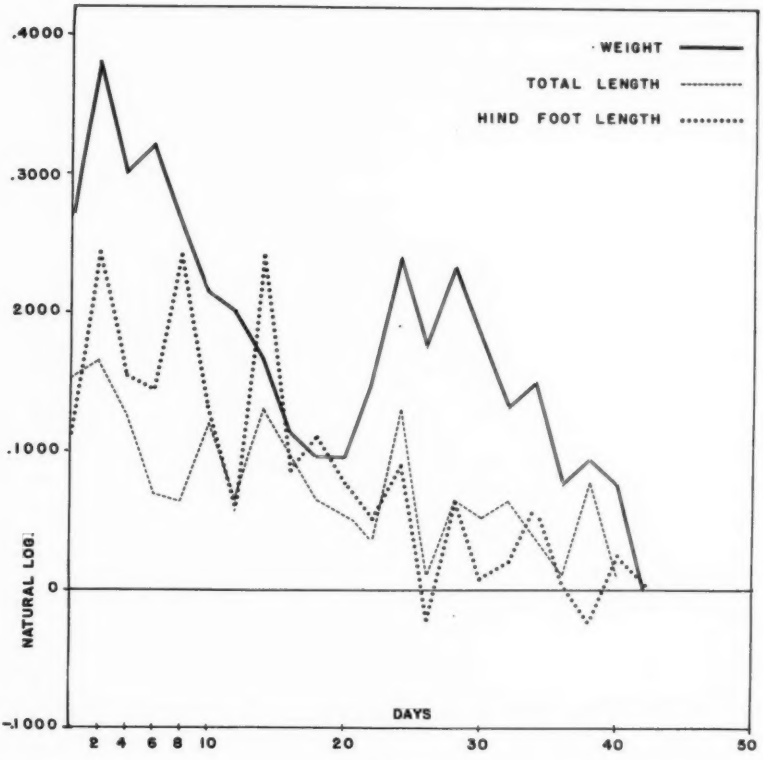


FIGURE 12.
Instantaneous growth rates in days of weight, total length, and hind foot length of litter of nine.

the hind foot attained a length of 54.4 millimeters, or 86.7% of the average adult hind foot length of 62.7 millimeters. By the 40th day the ear reached a length of 12.5 millimeters, or 84.4% of the average adult ear length of 14.8 millimeters. As the ear does not begin to enlarge significantly until about the eighth day, this size was attained within a growing period of 32 days. That the growth of these appendages is allometric can be seen from Figure 13.

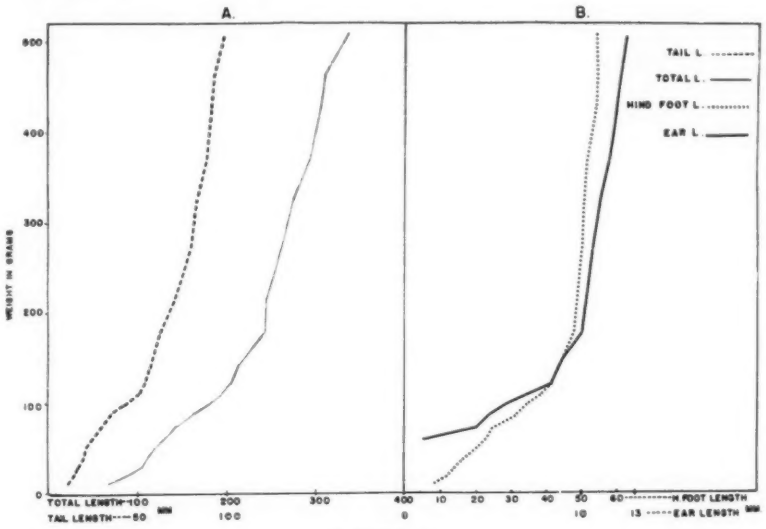


FIGURE 13

Allometric nature of growth of the litter of nine is demonstrated by plots of various lengths against weight. A smoother curve would probably have been obtained, it is believed, if a larger number of animals had been included.

According to Allen's Principle the appendages of northern forms are relatively shorter than those of their counterparts in temperate or tropical climates, the apparent reason being an adaptation allowing smaller amounts of exposed surfaces in the colder climates. When the appendages of *S. undulatus* are compared to those of *S. beecheyi* (Howell 2, p. 149), a California form nearly similar in size, Allen's Principle is found to hold good in the instance of their tail length, which is only 68.5% of that of *S. beecheyi,* and in ear length, 72% that of *S. beecheyi,* but not in hind foot length, which is 110%

that of the California squirrel. This exception is explicable, however, as another adaptation to the arctic environment. As the squirrels are active in the spring of the year, when snow still covers the ground, and also in the fall, when a similar situation may prevail, a large hind foot is beneficial for its snowshoe effect. This adaptation shows even greater development in certain other arctic forms such as the snowshoe hare.

Fitch (1, p. 580) indicates that the young of *S. beecheyi* do not reach the size at which they appear above ground until about 55 days of age, while young *Spermophilus undulatus* emerge from the natal burrow about a month after birth, appearing above the surface of the ground shortly after the middle of July. The early appearance above ground of the northern form appears to be adaptive, allowing maximum time for foraging and preparation for hibernation. Twenty-five *S. undulatus* young captured from July 17th to 20th, when first emerging from the natal burrow, averaged 218.8 grams in weight. The average weight of 51 young captured throughout the month of July was 261.2 grams. Of those sexed, 22 males averaged 250.2 grams and 15 females averaged 258.9 grams, or 8.7 grams more than the males (Figure 2).

Thirty-nine young squirrels weighed in August averaged 567.3 grams. Of those sexed, 20 males averaged 562.5 grams, and 15 females averaged 539.3 grams, or 23.2 grams less than the males. The weight increment for the total population of young from July to August was 117%.

In September the 12 young captured weighed an average of 880.3 grams, the seven males weighing 892.6 grams and the five females weighing 863.1 grams, or 29.5 grams less than the males. The average weight increment was 55% from August to September.

In October only four young males were captured. These weighed an average of 909.2 grams, representing an increase of 3.28% over the average September weight.

The preceding figures show August to be the month of greatest post-natal growth. This is further indicated by Figure 14, on which the monthly weight patterns have been plotted as instantaneous growth rates. Throughout the months of August and September together there is an average gain of 237% over the July weight, the males gaining 256.8% and the females 233.4%. This increment is sufficient to allow the young squirrels to approach adult weight by

the end of September, although they cannot fully attain the pre-hibernation weights of adults during their first summer, even by post-poning hibernation until October. The average weight of five male

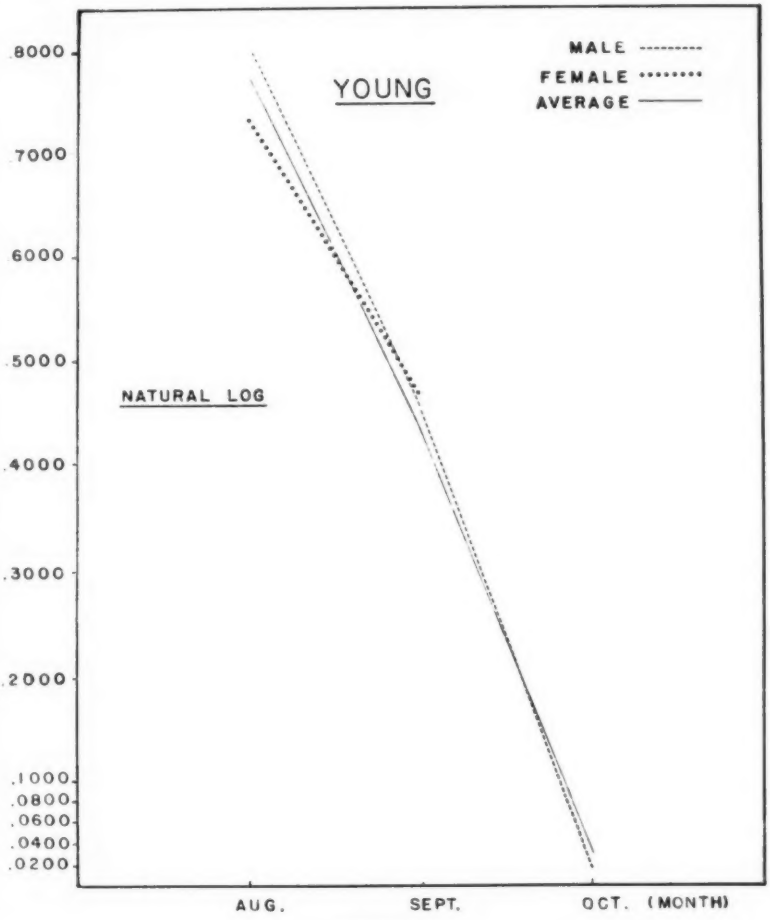


FIGURE 14
Changes in weight for young animals of the study area plotted as the natural log W_2 minus the natural log W_1 to give instantaneous growth rates for the months of surface activity.

adults in August was 13% or 118.2 grams more than the average of the four young males weighed in October. The pre-hibernation weights of young and adult females, however, showed less difference. The average weight of ten female adults captured in August was only 10.5 grams, or 1.2% more than that of five young females weighed in September. Despite the failure of the young to fully attain adult weight before hibernation, both the male and female young emerge in breeding condition the following spring.

Summary

The growth and developmental patterns of *Spermophilus undulatus barrowensis* are correlated with the arctic climate. Early emergence of the adults from hibernation while snow still covers the ground allows for mating in May, the animals existing during this time on stocks of stored food from the previous fall and what dead and frozen vegetation can be dug from beneath the snow. The young are born in June after a short gestation period of 25 days. They nurse for about one month and then appear above ground in July, when fresh, green vegetation is abundant. The young are born relatively well developed as compared to the young of other spermophiles, and they continue to grow at such a rapid rate that by their 42d day they have reached over 80% of adult size and 58.6% of adult weight. This rapid development allows them to take full advantage of the brief summer season and also frees the mother from caring for them at an early date, so that she can regain lost weight. After the breeding season the adults gain weight rapidly and are ready to enter hibernation in August. The young require an extra month to approximate adult weight, and most of them do not enter hibernation until September.

Thus an adaptive growth pattern allows the adults to mate, to give birth to young, and to prepare for hibernation between May and September. The young animals are able to attain approximate adult size and weight as well as able to prepare burrows and store food in the period from June to September, with an occasional animal still active in October. Within this brief growing season the young have also reached that point in development where they can emerge in the spring of the following year at their full reproductive potential.

Acknowledgments

The work on this paper represents the joint effort of both authors. The senior author outlined the scope of the paper and wrote it essentially as it appears. The junior author was responsible for the graphs and for checking and recalculating the data used in the text. The authors and another investigator, Mr. Roy G. Robinson, Jr. gathered data for this paper at a site on the Meade River, approximately 46 miles south of Point Barrow, Alaska, from May to October, 1952. This data has since been supplemented by observations on captive animals in the colony at the University of Southern California. Special thanks are due Mr. W. C. T. Yang who derived the data from which the logarithmic figures were made. This is contribution No. ONR 1953-11 from the Arctic Research Laboratory, Point Barrow, Alaska, and was supported by the Arctic Institute of North America and grant Nonr 377(00) from the Office of Naval Research.

References

1. Fitch, H. S. 1948. Ecology of the California ground squirrel on grazing lands. *Amer. Midl. Nat.*, **39**, 513-596.
2. Howell, A. H. 1938. Revision of the North American ground squirrels, with a classification of the North American Sciuridae. U.S.D.A. North Amer. Fauna No. 56, 256 pp.
3. Johnson, G. E. 1931. Early life of the thirteen-lined ground squirrel. *Trans. Kansas Acad. Sci.*, **34**, 282-290.
4. Mayer, W. V. 1952. Preliminary growth studies of the Barrow ground squirrel.. *Citellus parryi barrowensis*. *Anat. Rec.*, **113**, 79-80.
5. ———. 1953. Some aspects of the ecology of the Barrow ground squirrel, *Citellus parryi barrowensis*. Stanford Univ. Publ., Univ. Ser., *Biol. Sci.*, **11**, 48-55.
6. ———. 1953. A preliminary study of the Barrow ground squirrel, *Citellus parryi barrowensis*. *J. Mammal.*, **34**, 334-345.
7. ———. 1953. Acclimatization of the Barrow ground squirrel, *Citellus parryi barrowensis*. *Anat. Rec.*, **117**, 607.
8. Svihla, A. 1939. Breeding habits of Townsend's ground squirrel. Murrelet, January-April.
9. Shaw, W. T. 1925. Breeding and development of the Columbian ground squirrel. *J. Mammal.*, **6**, 106-113.
10. Wade, O. 1927. Breeding habits and early life of the thirteen-striped ground squirrel, *Citellus tridecemlineatus* (Mitchell). *J. Mammal.*, **8**, 269-276.